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Grau de Química

Treball Final de Grau

Triplex DNA structures in chemical and biochemical analysis

Estructures triples de DNA en anàlisi química i bioquímica

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The scientist is not the person who gives the right answers, but the one who asks the right questions.

Claude Lévi-Strauss

En primer lloc, m'agradaria donar les gràcies al meu tutor Raimundo Gargallo per la seva predisposició, el temps dedicat i l'aportació de coneixements durant aquest període de temps. Gràcies a la seva ajuda acadèmica, la realització d'aquest projecte ha estat un camí més fàcil.

Per últim, agrair als meus pares i amics per la paciència, els ànims i el suport proporcionat en tot moment per poder desenvolupar aquest treball.

REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

The Sustainable Development Goals (SDGs) consist of 17 universal objectives grouped into five different areas (called 5 Ps): People, Prosperity, Planet, Peace, and Partnership. These areas were accepted at the United Nations Sustainable Development Summit in 2015 and their primary aims are to eradicate poverty, protect the planet and ensure health and well-being for people. These goals are outlined in a new sustainable agenda called the 2030 Agenda which addresses social (peace and justice), economic (inequality), and environmental (climate change and sustainable consumption) challenges to promote human development sustainably.[1]

Since this project focuses on the analytical and bioanalytical applications of the DNA triple helix, it fit appropriately with 3 of the 17 goals:

SDG 3: Good health and well-being

Advances in DNA triple helix research are crucial for developing personalized medical treatments adaptable to individual genetic profiles. Additionally, gene therapy applications are capable of correcting mutations and preventing challenging genetic diseases, thereby enhancing people's health and well-being.

SDG 9: Industry, innovation and infrastructure

The distinctive properties of the DNA triple helix contribute to the development of innovative, precise, and accessible technologies that enable its application in the biotechnology and pharmaceutical industry.

SDG 4: Quality education

The advancements enabled by the triple helix in recent years can enhance quality education in the fields of genetics and biotechnology, inspiring new generations to participate in scientific research developing their knowledge and skills.

CONTENTS

1. SUMMARY

The DNA triple helix represents an alternative structure compared to the traditional DNA double helix, owing to the molecule's flexibility to adopt a wide variety of helical structures. Over the past years, the formation and stability of triple helix structures have demonstrated significant applications in multiple scientific disciplines.

This work presents a bibliographic research focus on the triplex DNA structure, the factors which determine its stability and its relevance in chemical and biochemical analyses. Additionally, several analytical techniques that enable the detection of triplex DNA structures are also examined, including molecular absorption spectroscopy and electrophoretic techniques.

Its structure relies on the specific binding of a triplex-forming oligonucleotide (TFO) to a specific sequence of a double-stranded DNA molecule through Hoogsteen base pairs. This research provides an analysis of the functionality of Hoogsteen and reverse Hoogsteen hydrogen bonds in the stabilization of parallel and antiparallel triplex structures, respectively. For this reason, pH is a critical factor that can enhance or limit the stability and potential applications of the DNA triple helix.

One of its most important analytical applications is the detection and recognition of specific biomolecule sequences with high sensitivity and selectivity, as well as the consequent development of advanced biosensors. Furthermore, the use of the triple helix in the fields of nanotechnology and biotechnology through the fabrication of nanodevices has also been examined. Additionally, the DNA triple helix has emerged as a promising platform for gene therapy, providing new avenues for the correction of specific mutations, regulation of gene expression and diagnosis of genetic and cancerous diseases.

Keywords: DNA triple helix, triplex-forming oligonucleotide, stability, pH, biosensors, nanodevices, gene therapy

2. RESUM

La triple hèlix d'ADN representa una estructura alternativa a la tradicional doble hèlix d'ADN, gràcies a la flexibilitat que presenta la molècula per adoptar una gran varietat d'estructures. Durant els últims anys, la triple hèlix ha demostrat tenir aplicacions de gran interès en diferents disciplines científiques.

En aquest treball s'ha realitzat un estudi bibliogràfic centrat en l'estructura de la triple hèlix d'ADN, així com els factors que determinen la seva estabilitat i la seva rellevància en anàlisis químiques i bioquímiques. També s'analitzen diferents tècniques analítiques que permeten la detecció de les estructures d'ADN tríplex, incloent tant l'espectroscòpia d'absorció molecular com tècniques electroforètiques.

La seva estructura ha demostrat estar basada per la unió específica d'un oligonucleòtid formador de tríplex (TFO) amb una seqüència específica d'una molècula d'ADN de doble hèlix mitjançant parells de bases Hoogsteen. Aquesta investigació proporciona un anàlisi de la funcionalitat dels enllaços d'hidrogen Hoogsteen i Hoogsteen invers en l'estabilització de les estructures de tríplex paral·lel i antiparal·lel, respectivament. Per aquesta raó, el pH jugarà un paper molt important i potenciarà (o limitarà) l'estabilitat i les potencials aplicacions de la triple hèlix.

Una de les aplicacions analítiques més destacades consisteix en la detecció i reconeixement de seqüències específiques de biomolècules amb alta sensibilitat i selectivitat, així com el conseqüent desenvolupament de biosensors avançats. També s'ha examinat el seu ús en l'àmbit de la nanotecnologia i la biotecnologia mitjançant la fabricació de nanodispositius. Paral·lelament, la triple hèlix d'ADN també es proposa com una plataforma prometedora per a la teràpia gènica, proporcionant noves vies per a la correcció de mutacions específiques, la regulació de l'expressió gènica i el diagnòstic de malalties genètiques i canceroses.

Paraules clau: triple hèlix d'ADN, oligonucleòtid formador de tríplex, pH, estabilitat, biosensors, nanodispositius, teràpia gènica

3. INTRODUCTION

Deoxyribonucleic acid (DNA) molecules are right-handed helices composed of two antiparallel nucleotides strands. Nucleotides consist of three different subunit molecules: a five-carbon sugar, a nitrogenous base and a phosphate group. The four distinct types of nucleobases (adenine, guanine, cytosine and thymidine or uracil), which are the foundation of the genetic code, are connected by hydrogen bonds forming base pairs along the axis of the helix. Thus, the double helix structure is maintained by the formation of hydrogen bonds between the bases of DNA, specifically the adenine-thymine pairs and the cytosine-guanine pairs.[2]

In biological systems, DNA typically adopts a double helix structure. However, DNA is highly flexible and can adopt a large variety of helical structures such as triple and quadruple helices depending on the sequence, ionic environment, temperature, solvent, or the presence of ligands.[3]

3.1. TRIPLEX DNA FORMATION BY OLIGONECLEOTIDES

The discovery of triple-stranded DNA (tsDNA) dates back to 1959, when Karst Hoogsteen described a base-pairing variation from the Watson-Crick model known as Hoogsteen base pair (Figure 1). This model defines the formation of triplex structures where a single-stranded triplex-forming oligonucleotide (TFO) specifically binds to the major groove of Watson-Crick homopurine (not the pyrimidine) double-stranded DNA through Hoogsteen hydrogen bonds.[4]

A Hoogsteen base pair can occur between A·T bases under neutral conditions, specifically applying the N3 position of 1-methylthymine linking to N7 of 9-methyladenine, while the carbonyl group C4 of 1-methylthymine forms a hydrogen bond with the amino group of 9-methyladenine. However, if cytosine is protonated, it can also occur in G·C$^+$ pairs. Purine bases have sufficient groups for hydrogen bond formation with their complementary strand (Watson-Crick pairings) and with another chain of complementary bases (Hoogsteen pairing).[5]

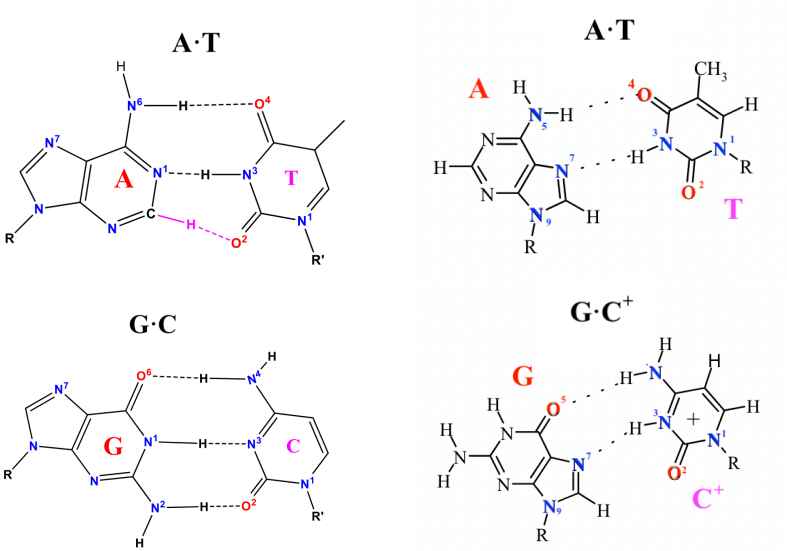

Figure 1: Comparison between Watson-Crick (left) and two examples of Hoogsteen (right) base pairing

The stability of triplexes is influenced by four primary factors: the inherent stability of nucleotides, phosphate repulsions, stacking interactions and Hoogsteen hydrogen bonds. Consequently, triplexes achieve greater stability in a high ionic strength environment capable of reducing the repulsion of the negative charges of the DNA strands, specifically the phosphate-phosphate repulsion between the duplex and the third strand. The stability can be also enhanced when nucleotides adopt stable conformations or minor tautomeric forms and by the presence of a proper arrangement of hydrogen bonds and stacking interactions.[3]

Depending on the orientation of the TFO relative to the central oligopurine Watson-Crick strand, triplex structures are generally categorized into parallel and antiparallel (Figure 2).

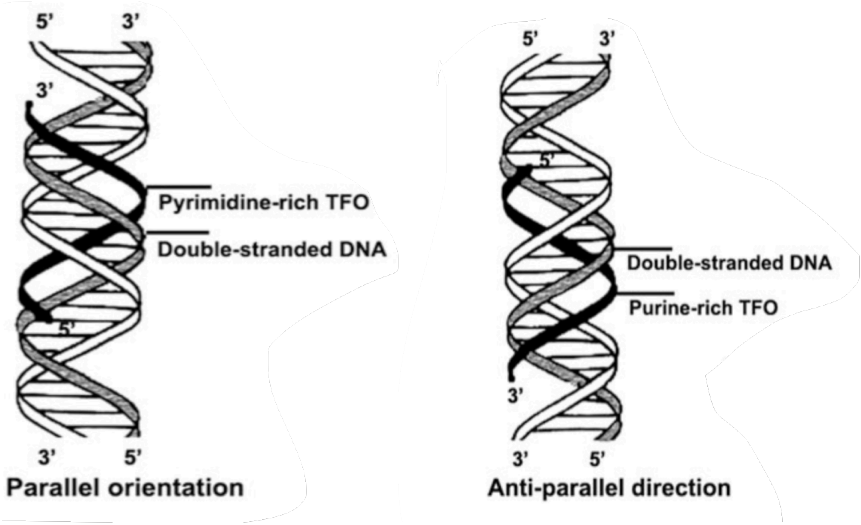

Figure 2: Parallel and antiparallel conformations that tsDNA structure can adopt (image reprinted with permission from reference 6)

3.1.1. Parallel triplexes

In parallel triplexes (Figure 3), also named pyrimidine-triplexes, the TFO is made of C and T residues (C,T-TFO) and it binds parallel to the homopurine stranded Hoogsteen base pairs forming T·A:T (in this notation an "·" represents third strand binding and a ":" the Watson-Crick base pairing of the target duplex) and C^{+}·G:C triads (Figure 4). The protonation of cytosine provides stability to the parallel triplex which depends on pH, being more stable at an acidic pH near the pKa value of cytosine (pKa = 4,5).[4]

It has been observed that the isomorphic C^{+}·G:C triad can be substituted by the G·G:C triad (Figure 4), not isomorphic one. In this case, the TFO is formed by G and T residues (G,T-parallel triplex) where the stability of the triplex does not depend on pH because protonation is not required. Whenever there is a transition from a T·A:T triad to a G·G:C triad, the stability of parallel triplex decreases, making this type of triplex absent in mixed A,G sequences.[7]

Parallel triplexes receive a lot of attention from a structural and biochemical perspective because they exhibit a clear hypochromic shift (observable in UV melting curves) during triplex formation. This characteristic allows the study of the stability of these parallel triplexes.[4]

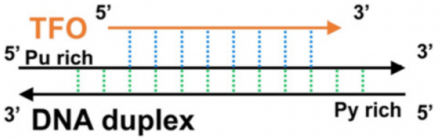

Figure 3: Strands orientation of parallel triplex with their respective Watson-Crick (green) and Hoogsteen (blue) base pairing

(image reprinted with permission from reference 8)

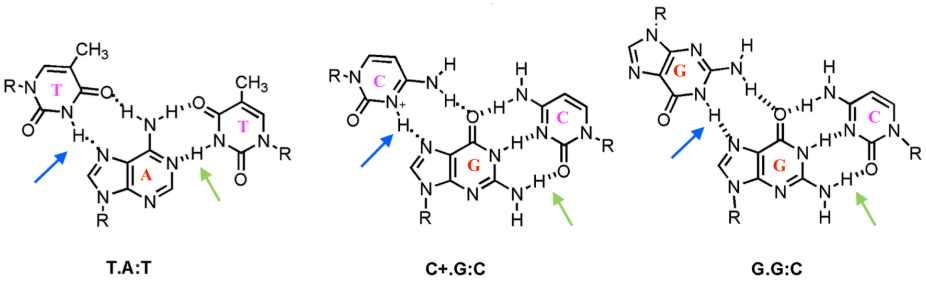

Figure 4: Chemical structures of possible parallel triads with their respective Watson-Crick (green arrow) and Hoogsteen (blue arrow) base pairing

(image reprinted with permission from reference 9)

3.1.1.1. Stabilization of parallel triplex by modified nucleobases

A notable problem lies in the necessity for protonation of cytosine residues in the Hoogsteen strand. In order to increase the stability of parallel triplex structures under physiological (neutral pH) conditions, significant efforts have been focused on advancing modified nucleobases.[10]

The use of 5-methylcytosine as a substitution for cytosine in TFO represents one of the earliest modifications to stabilize parallel triplexes by increasing pKa level and entropy due to stronger stacking interactions and helix desolvation. Similarly, the substitution of thymidine by 5-bromouracil also contributes positively.[11] Conversely, the studies conducted by E. Ferrer et al. have demonstrated that the substitution of other pyrimidines at position 5, such as 5-iodouracil, 5-bromocytosine, 5-iodocytosine and 5-aminouracil, results in negative effects on the stability of parallel triplexes.[12]

Substitution of thymidine by 5-propynyl-uracil significantly enhances the formation of the parallel triplex with dsDNA due to its hydrophobic properties which facilitate both desolvation and stacking interactions. However, the replacement of cytosine by 5-propynyl-cytosine does not

contribute to the stability of the triple helix but it stabilizes the double helix formed by a DNA strand and an RNA strand (oligodeoxynucleotides composed of 5-(1-propynyl)-2-deoxyuridine and 5-(1-propynyl)-2-deoxycytidine).[13]

3.1.2. Antiparallel triplexes

In antiparallel triplex (Figure 5), also named purine-triplexes, the TFO is made of G and A residues (G,A-TFO) and it binds antiparallel to the homopurine strand through reverse Hoogsteen base pairs forming A·A:T and G·G:C triads (Figure 6). Unlike parallel triplexes, in purine-triplexes there is no need for base protonation and their stability is pH-independent. This makes these triplexes more appropriate for their use in conditions similar to physiological ones.[4]

It has been described that the A·A:T triad can be replaced by the T·A:T one (Figure 6). In this case, the TFO is composed of G and T residues (antiparallel G,T-TFO) and is also pH-independent. Generally, it is slightly less stable than the G,A-antiparallel triplex.[14]

In contrast to parallel triplexes, the formation of antiparallel triplexes does not show hypochromic effects in UV spectra of DNAs. This lack of hypochromicity in purine-triplexes complicates the analysis of the relative stability using UV spectrometry and, as a consequence, there is much less data on literature on this type of triplex. However, the binding of antiparallel TFO to the duplex target can be observed and measured through other techniques such as gel foot-printing assays, fluorescent FRET analysis, resolution of melting curves by multivariate resolution and UV-Vis denaturation studies of oligonucleotide-gold conjugates.[9]

Parallel triplexes are more stable compared to their antiparallel counterparts under near-physiological conditions. Antiparallel triplexes exhibit a strong dependence on the surrounding counterion environment and require divalent ion high concentrations for their stability, which can lead to strong phosphate-phosphate repulsion that is reduced by the screening effect of cations.[3]

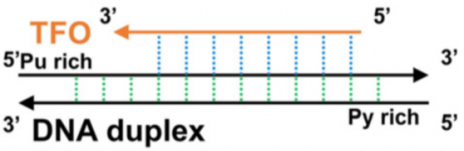

Figure 5: Strands orientation of antiparallel triplex with their respective Watson-Crick (green) and Hoogsteen (blue) base pairing

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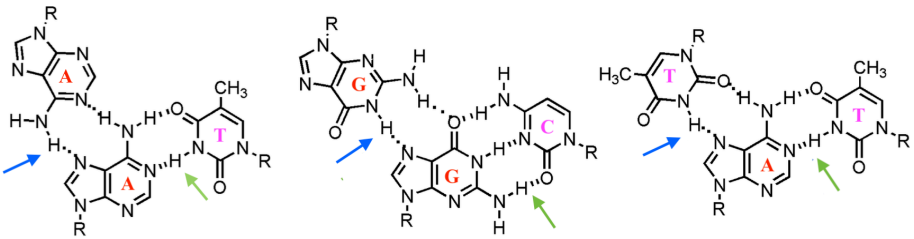


Figure 6: Chemical structures of possible antiparallel triads with their respective Watson-Crick (green arrow) and Hoogsteen (blue arrow) base pairing

(image reprinted with permission from reference 9)

3.2. STRAND DISPLACEMENT OF SINGLE-STRANDED AND DOUBLE-STRANDED DNA BY TRIPLEX FORMING USING HAIPINS OR CLAMPS

There are folded-back oligonucleotides known as clamps or hairpins which bind single-stranded nucleic acids with high affinity through triplex formation. In certain instances mentioned later on, the possibility of binding between the clamps and double-stranded DNA (dsDNA) has also been demonstrated.

3.2.1. Targeting the polypurine strand

The most extensively studied strategy for triplex formation using clamps targets the polypurine segment. This involves connecting the Hoogsteen polypyrimidine segment with the Watson-Crick polypyrimidine, creating clamps synthesized using phosphoramidites in solid-phase synthesis.[9]

Phosphoramidites are modified nucleosides with chemical protection groups on their reactive sites in order to prevent unwanted reactions during synthesis. The protective groups can be selectively removed during the synthesis process to enable specific reactions.

The strong affinity of clamps for their target sequence enables them to bind to double-stranded DNA, displacing the natural complementary Watson-Crick polypyrimidine segment due to the clamps stronger binding properties (Figure 7).[15]

The strategy of these clamps is also used with Peptide Nucleic Acids (PNAs). In the formation of the triplex, two molecules of homopyrimidine PNA (bis-PNA) are engaged, demonstrating a high affinity for homopurine DNA targets. This results in the formation of PNA·PNA·DNA triplexes, which have the capability to displace the Watson–Crick polypyrimidine segment.[16]

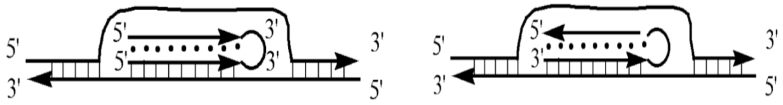


Figure 7: Parallel (left) and antiparallel (right) clamps targeting dsDNA targets producing strand displacement

(image reprinted with permission from reference 9)

3.2.2. Targeting the polypyrimidine strand

Another strategy for triplex formation is targeting the polypyrimidine strand, which has been achieved using two different families of folded-back triplex-forming oligonucleotides.

On the one hand, in parallel clamps linking the reversed Hoogsteen polypurine strand with the Watson–Crick polypyrimidine strand involves forming 3'-3' or 5'-5' internucleotide bonds (Figure 8). The synthesis of parallel clamps involves assembling one of the strands in the reverse direction (5'-3') using 5'-phosphoramidites. An alternative approach to prepare parallel clamps (without relying on 5'-phosphoramidites) is to synthesize both polypurine and polypyrimidine strands on separate supports and then combine them using the Cu(I)-catalyzed cycloaddition reaction.[17]

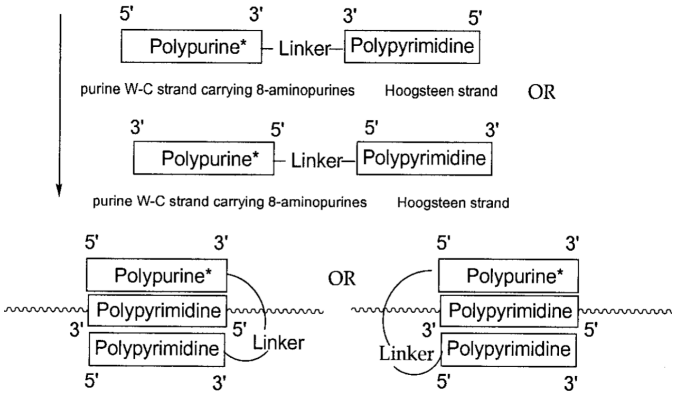


Figure 8: Diagram illustrating the interaction between a polypyrimidine ssDNA and the studied hairpins

(image reprinted with permission from reference 18)

Parallel-strand hairpins with a polypyrimidine sequence linked to a complementary purine containing 8-aminopurines (such as 8-aminoadenine, 8-aminoguanine, and 8-aminohypoxanthine) bind through the purine segment to complementary polypyrimidine sequences (in an antiparallel sense) forming a triple helix. Introducing an amino group at the 8-position of adenine and guanine enhances the stability of parallel-stranded hairpins under neutral conditions and the capability to manage interruptions in the polypyrimidine target sequences. It has been demonstrated that 8-amino-guanine exhibits a stronger stabilizing effect than 8-amino-adenine.[18]

The strategy of these clamps is also used with Peptide Nucleic Acids (PNAs). In the formation of the triplex, two molecules of homopyrimidine PNA (bis-PNA) are engaged, demonstrating a high affinity for homopurine DNA targets. This results in the formation of PNA·PNA·DNA triplexes, which have the capability to displace the Watson–Crick polypyrimidine segment.[13]

These modified hairpins have a higher affinity for binding to the Watson-Crick pyrimidine strand in a triple helix compared to hairpin consisting exclusively of natural bases. Thus, natural oligonucleotides with 8-aminopurines cannot be used directly for the specific binding of double helix DNA sequences. Furthermore, oligonucleotides carrying 8-aminoguanine also produce a high stabilization of antiparallel triplexes.[18]

On the other hand, it is important to consider Polypurine Reverse Hoogsteen (PPRH) strategy. Polypurine Reverse Hoogsteen (PPRH) molecules are hairpins composed of two polypurine strands linked through intramolecular reverse Hoogsteen bonds where guanines pair with guanines and adenines pair with adenines, oriented in an antiparallel manner and devoid of nucleotide modifications. The PPRH oligonucleotides have the capability to bind via Watson-Crick bonds to their respective polypyrimidine targets within double-stranded DNA and form triplexes commonly known as "purine motif triplexes". Their formation necessitates the presence of divalent cations, like Mg^{2+}, and exhibits relatively pH-independent characteristics.[19]

Studies have revealed that PPRHs play a role in forming three-stranded structures even in scenarios where they adopt G-quadruplex configurations (Figure 9).[9] When designing a PPRH motif rich in consecutive guanines, it is important to consider the potential formation of a guanine-quadruplex (G4) structure linked by hydrogen bonds, whose stability depends on the specific cation present in the solution, typically potassium ions.

Consequently, the behavior of PPRH is dependent on its sequence, as the folding of a PPRH oligonucleotide into a reverse Hoogsteen hairpin doesn't always result in a stable triplex, while under alternative conditions, PPRH folding can induce a transition from a stable G4 structure to a reverse Hoogsteen hairpin, facilitating the formation of a stabilizing triplex with its single-stranded polypyrimidine target.[19]

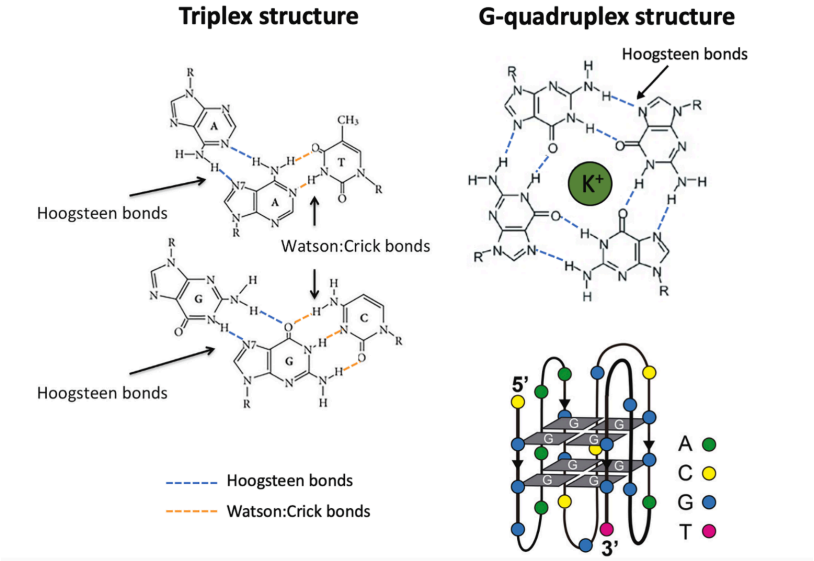


Figure 9: Chemical representation of Hoogsteen and Watson Crick base pairing that participate in the formation of triplex or G-quadruplex structures

(image reprinted with permission from reference 20)

A key concern in parallel hair clamps and PPRH design is the presence of interruptions in the target polypyrimidine sequence. Suggestions include using the complementary base in the Watson-Crick strand, using cytosine in the Hoogsteen strand for guanine interruptions and using guanine in the Hoogsteen strand for adenine interruptions. In PPRH, recommendations involve adding an adenine at the Watson-Crick position and another adenine at the Hoogsteen position or maintaining the pyrimidine interruption within the PPRH sequence.[9,19]

Apart from clamps, other oligonucleotide DNA structures have been described such as cyclic oligonucleotides, wedge and tailed clamps. In each of these instances, increased affinity for the polypyrimidine target and increased specificity have been reported.[9]

Regarding to tailed-clamps, the efficiencies of union are higher in anti-parallel tail-clamps compared to parallel tail-clamps, particularly in neutral and basic pH conditions. Furthermore, some studies show that both parallel and antiparallel tail-clamps efficiently bind to complementary single-stranded RNA oligonucleotides, forming triple helix structures.[21]

3.3. APPLIED TECHNIQUES TO STUDY TRIPLEX DNA STRUCTURES

There are different techniques to study the formation, stability and structure of DNA triple helices. Below are presented some of the most applied techniques to study and detect the formation of DNA structures: molecular absorption spectroscopy, circular dichroism (CD) spectroscopy, polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis (CE).

3.3.1. Spectroscopic techniques

Firstly, a widely used technique that can be useful to detect the formation of DNA triple helix structures is UV-Vis Spectrophotometry. As mentioned previously, parallel triplexes (similar to duplexes) exhibit a significant hypochromic effect, meaning that the absorption of UV light is lower compared to the sum of the absorptions of the individual strands due to base stacking and hydrogen bonding. However, in antiparallel triplexes, the change is much smaller creating more limitations on their structural study.[4]

DNA absorbs light in the ultraviolet and visible region of the electromagnetic spectrum because of its aromatic nitrogenous bases. UV-Vis spectroscopy is a valuable technique that allows the observation of changes in the absorbance spectrum of DNA samples, reflecting alterations in the arrangement of nitrogenous bases and the three-dimensional structure upon the formation of triple helix.[22]

Secondly, in CD spectroscopy the process involves detecting the difference in absorption between left and right circularly polarized light in optically active substances. Consequently, chiral samples generate effective signals. It is a useful and rapid method for studying DNA structures, reflecting extremely sensitively the isomerizations between different conformational states.

Generally, the CD signals which indicate relative orientations from electronic transitions of the bases between 200-320 nm are tracked.[23]

Regarding the spectroscopic results, the breakpoint on the mixing curve in CD-recorded spectra of Figure 10B, indicates the presence of $C^{+}\cdot C$ pair bases and the consequent formation of the parallel triplex (acidic pH levels at 5.6) monitored at 280-300 nm, which is not present in CD-recorded spectra of the duplex (Figure 10A). Moreover, the presence of a negative minimum around 240-250 nm in the triple formation (Figure 10B) due to the greater interaction between the stacked bases is also absent in duplex formation (Figure 10A). Another characteristic region in triplex formation around 200-210 nm (Figure 10B) shows additional maximums and minimums which represent interactions among the three nucleotide chains and reflect the added complexity of the triple helix.[24]

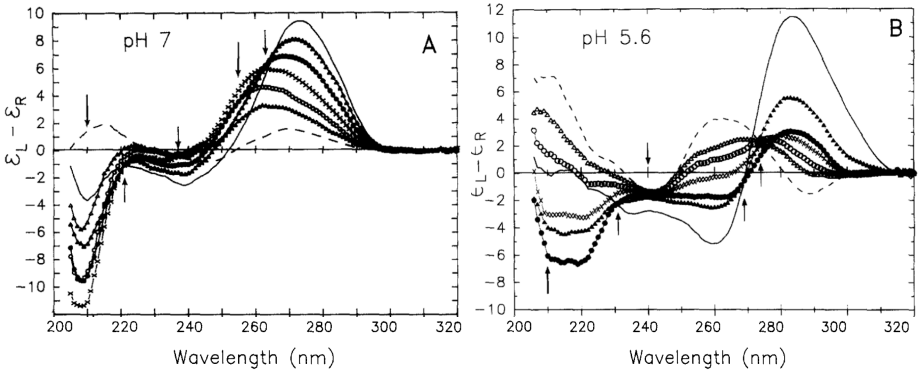


Figure 10: CD spectra of (A) a duplex DNA formation containing samples of $d(AG)_{12}$ and $d(CU)_{12}$ and (B) a parallel triplex DNA formation containing samples $d(AG)_{12}$ and $d(CT)_{12}$, at different molar proportions under experimental conditions (50 mM sodium phosphate buffer, 20ºC)

(image reprinted with permission from reference 24)

As demonstrated by the authors Callahan, D. et al., the oligodeoxyribonucleoside methylphosphonates are nucleic acid analogs that contain non-ionic methylphosphonate internucleoside linkages, in contrast to the naturally present negatively charged phosphodiester linkages. The CD assays reveal that triplex formation occurs at a higher pH level, enabled by negatively charged phosphodiester backbones and advantageous interactions between protonated cytosines.[25]

Recent studies by the authors Quartin, R. and Wetmur, J. have demonstrated that oligodeoxyribonucleotides modified with methylphosphonate have the capability to bind to single-stranded DNA. The resulting hybrid duplexes (which contain one phosphodiester chain and one methylphosphonate chain) exhibit modified stability in comparison to phosphodiester duplexes.[26] Simultaneously, the authors Miller, P. et al. revealed that each methylphosphonate linkage forms one of the two stereoisomers (R or S configuration), resulting as a mixture of diastereoisomers.[27] The observed changes in the corresponding CD-spectra are valuable for monitoring the transition from duplex to triplex conformation.[28]

Unusual properties such as abnormally high "helical twist angles" have also been studied in circular dichroism studies for oligonucleotides containing adenine and thymidine sequences. This phenomenon may stem from adenine forming a bifurcated hydrogen bond, engaging with a thymidine directly opposite to it, as well as with thymidines positioned above or below it.[29]

3.3.2. Electrophoretic techniques

On the other hand, polyacrylamide gel electrophoresis (PAGE) is a highly effective technique to separate DNA mixtures based on their molecular sizes. High-concentration polyacrylamide gels are commonly employed as the separation medium establishing a molecular sieve. Under the influence of an electrical field, molecules traverse through the gel with a velocity inversely proportional to their lengths.[30] However, the intermolecular tsDNA structure is more easily determined using capillary electrophoresis (CE) than the traditional slab gel electrophoresis techniques.

In recent years, CE has made a significant impact on analytical chemistry due to its ability to achieve high-resolution separation using minimal sample volumes, seamless integration with automated sample handling and automatic on-line determination of triplexes, simplifying method validation.[31] There are important DNA online-detection methods such as UV absorbance detection and laser-induced fluorescence detection. The ease of online detection has made CE much more suitable for automation than plate gel electrophoresis.[2]

Electrophoretic mobility exhibits sensitivity to certain experimental conditions such as temperature. At temperatures above 30°C, the formed triplex dissociates completely. Thus, the optimal selected temperature for the capillary column in the separation is 15°C (Figure 11). Inadequate cooling of the capillary can result in the development of a parabolic electrophoretic

velocity profile, ultimately causing band broadening. To maintain capillary temperature, forced air convection at a controlled temperature is commonly used.[2] On the other hand, existence of transition metal cations is crucial for the creation and stabilization of the triplex due to the DNA's particular binding sites for divalent cations capable of stabilizing both double-stranded DNA and triple-stranded DNA.[32]

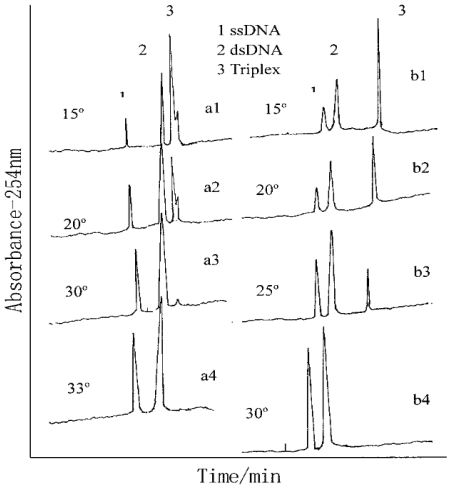


Figure 11: Electropherogram of triplex-containing samples of A_{20} and T_{20} (a1-a4) and dsDNA with TFO (b1-b4), at different column temperatures. Separation conditions: 1.0% HPMC sieving matrix, pH 8.5, UV detector at 254 nm, 9-15 kV running voltage, 10 kV electrokinetic injection

(image reprinted with permission from reference 31)

From the initial studies on capillary gel electrophoresis of proteins with SDS (SDS-CGE) developed by authors Hjerten, S. and Karger, B. it's becoming evident that nearly all techniques originally designed for slab gel electrophoresis can seamlessly transition to a silica capillary setup. This shift presents notable benefits such as fully automated processes and swift and superior resolution analysis.[33,34]

The use of a fused silica capillary column is the primary contrast between PAGE and CGE. In CGE, users traditionally filled gel capillaries with cross-linked polyacrylamides, renowned for their efficacy in analyzing DNA and protein molecules in the presence of the anionic detergent SDS. Recently, prefilled gel capillary columns are offered by an expanding number of manufacturers.[30]

To conclude, SDS-CGE represents an enhanced instrumental method compared to SDS-PAGE providing a rapid and automated separation achieving on-column injection, detection and quantification without additional time and labor demands.

4. OBJECTIVES

The aim of this project is to do bibliographic research of the latest applications of DNA triplex structures in chemical and biochemical analysis. In order to carry out the purpose, the main topics are as follows:

(1) Firstly, it is necessary to investigate the physical and chemical properties of DNA triplex structures as well as the mechanisms and optimal conditions for its formation.

(2) Exploring key factors that influence its stability and some potential modifications in nucleobases that allow stabilizing the structure of triple helices.

(3) Determining the importance of triplex DNA structures detection and studying some spectroscopic and electrophoretic techniques involved in the formation of DNA triple helix structures.

(4) Studying the potential properties and applications of DNA triple helices in chemical biosensing, molecular probes and nanotechnology.

Initially, the first three points have been presented on the previous pages and the fourth and final point is presented on the following ones.

5. METHODS

Various databases and platforms accessible through CRAI-UB services have been used in this bibliographic research about DNA triplex structures, including the American Chemical Society (ACS) database, Web of Science, SciFinder, SpringerLink and ScienceDirect. It should be noted that the figures used in this work have been reused with permission from the Copyright Clearance Center.

Initially, my tutor provided me with several scientific articles through the Mendeley platform to familiarize myself with triplex structures, which allowed me to discover many other articles through their bibliographies to continue the research.

Subsequently, the main search criterion used has been specific keywords related to the triple helix of DNA. Based on the results obtained and focusing on the previously established objectives, additional filters have been applied to limit the search by factors such as stability, detection techniques, biosensors... Generally, the scientific reports consulted were limited to the last 10-15 years.

Regarding the previously mentioned Mendeley platform, it has been very helpful for storing and organizing all kinds of information sources. Some of its main features used include importing articles from other research programs, searching for documents based on previous readings and the automatic generation of bibliography.

6. RESULTS AND DISCUSSION

In recent years, the formation and stability of triple DNA helices have developed a wide variety of significant applications in the fields of biomedicine and biotechnology. Therefore, these unique DNA structures continue to be an active area of research with great potential.

6.1. PPRH HAIRPINS AS DETECTION MOLECULES: SURFACE PLASMON RESONANCE

PPRH hairpins are used as versatile tools for molecular detection due to their ability to form specific structures and bind to complementary sequences. Recently, the Surface Plasmon Resonance (SPR) optical biosensor methodology based on PPRH probes has been developed for potential applications in cancer research, early disease diagnosis, and personalized therapy development.[20]

SPR technology works by detecting the biomolecular interaction between a target analyte and its capture receptor, which is immobilized on a gold surface. The recognition of the target molecule induces a change in the refractive index proportional to its concentration on the sensor surface, which is detected through a change in reflectivity at the SPR sensor interface (Figure 12).[35]

The DNA methylation state is a crucial cancer biomarker for both diagnosis and patient monitoring. Through the formation of triple-helix structures, PPRH probes are anchored onto the biosensor surface and exposed to biological samples containing hypermethylated DNA fragments. Consequently, these probes can selectively bind to these fragments and generate interactions that induce changes in the intensity of the reflected light, which are related to the amount of molecules binding to the sensor surface, thereby determining the presence and quantity of hypermethylated DNA fragments in the sample.[36]

PPRHs have also been used for the rapid detection and diagnosis of pneumonia in human samples which currently poses a challenge due to the absence of specific symptoms in infected patients. The PPRH probe exhibits exceptional selectivity and reproducibility in detecting the mtLSU rRNA gene of the *Pneumocystis jirovecii fungus*, the causative agent of pneumonia, achieving detection limits of approximately 2.11 nM. This leads to a shift in the wavelength of reflected light, enabling the quantification of the analyte bound to the SPR.

By reducing diagnosis time, this methodology could enable an early clinical response, impeding disease progression and reducing mortality rates. For this reason, it could emerge as the preferred diagnostic tool for Pneumocystis pneumonia.[37]

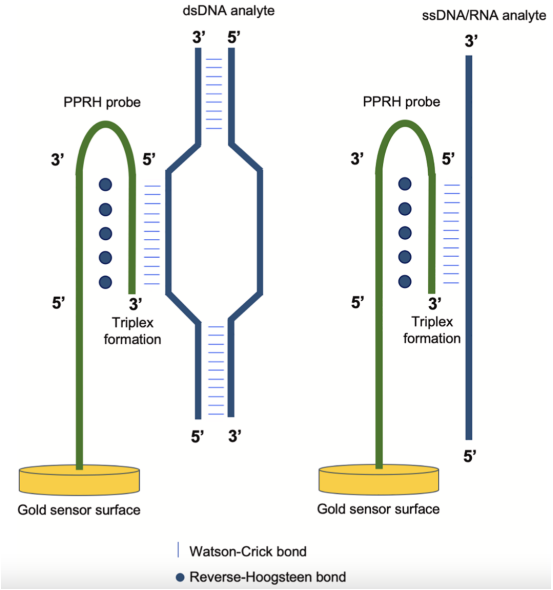


Figure 12: A schematic representation of biosensors utilizing PPRHs as probes to detect specific dsDNA fragments (left) or ssDNA/RNA (right) through triplex helix formation

(image reprinted with permission from reference 20)

In that way, the combination of SPR biosensors and PPRH technology provides a powerful means for real-time detection of specific biomolecular interactions with potential applications in biomedical research, clinical diagnosis, and drug development.[20]

6.2 SENSITIVE AND LABEL-FREE DETECTION OF miRNA

MicroRNAs (miRNAs) are short RNA molecules that play an important role in fundamental biological processes, including apoptosis and the regulation of gene expression at the post-transcriptional level. Due to the low levels of miRNAs in human cells, detection methods need to be highly sensitive and selective with small RNA sample quantities.

Although various methods exist for miRNA detection, many of them require large sample volumes and are not always precise in measuring miRNAs. Therefore, developing new strategies for miRNA detection has become a critical step in the field of diagnostics.[38]

A strategy has been developed for detecting miRNA-145 using SPR based on the formation of stable triple-helix structures. This miRNA functions as a tumor suppressor with reduced expression observed in most colon and gastric cancer cells.

Focusing the SPR technology on the newly developed approach, DNA bioreceptors (tail clamp bioreceptors carrying 8-aminoguanines) immobilized on the gold biosensor surface have been used to target and interact with specific nucleic acid sequences (miRNA-145). When miRNA detection occurs, the expected variations in the refractive index of the gold surface are generated due to miRNA hybridization, consequently changing the intensity of the reflected light. It is important to note that detecting miRNA-145 is challenging because they are small RNA molecules, and the Hoogsteen polypyrimidine tracks are shorter (7 nucleotides), which could affect the stability of the triplex. For this reason, 8-aminoguanine is used instead of 8-aminoadenine and parallel and antiparallel tail-clamps to enhance triplex stability.[10,18]

UV denaturation curves and CD measurements have confirmed that the parallel tail clamps modified with 8-aminoguanine form the most stable triple-helix structures at neutral pH with their target miRNA-145. The appearance in CD spectra of an intense negative band near 210 nm indicates the formation of a triplex (Figure 13). These results provide a new and sensitive approach for detecting miRNA-145 using an SPR biosensor.[38]

The findings highlight the significant potential of triple-helix structures in the miRNA field, particularly for the detection of miRNAs for diagnostic and biomedical purposes.

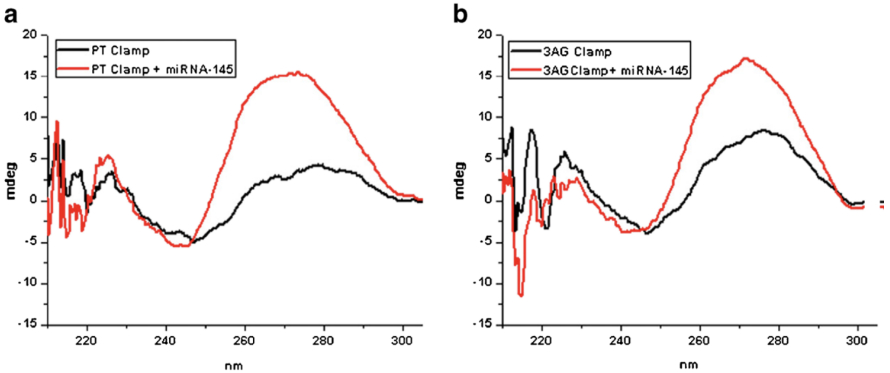


Figure 13: CD spectra of (a) tail clamp (PT) alone and with miRNA-145 (b) and tail clamp with 8-aminoguanine (3AG) alone and with miRNA-145, at pH 6 and 100 mM phosphate buffer
(image reprinted with permission from reference 38)

6.3. DETECTION OF SARS-CoV-2 VIRUS BY TENADA

The COVID-19 pandemic has led to significant efforts to create effective techniques for detecting the SARS-CoV-2 virus and implementing appropriate measures to prevent its transmission. The standard method for diagnosing COVID-19 is polymerase chain reaction (PCR). However, it demands specialized staff and equipment and takes several hours to finish. Therefore, the need for quicker solutions has driven the development of alternative methods for the rapid detection of SARS-CoV-2.[39]

Building on the successful results achieved in detecting miRNA with DNA hairpins through triplex formation using SPR, researchers have analyzed the SARS-CoV-2 genome and have discovered numerous polypyrimidine sequences that can bind to triplex-forming DNA hairpins, enabling direct detection of viral RNA without the need for PCR.[40]

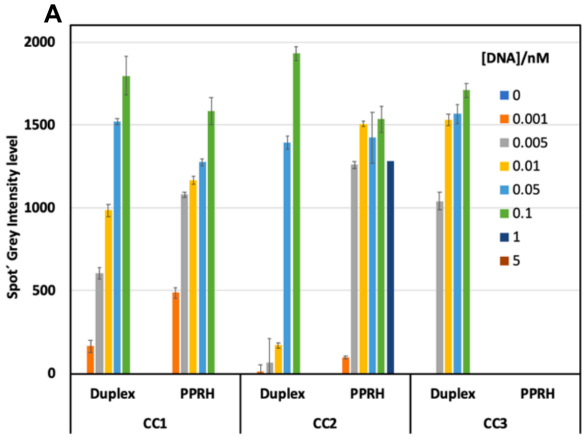
In this way, using the method known as Triplex Enhanced Nucleic Acid Detection Assay (TENADA), the presence of SARS-CoV-2 RNA is detected through the formation of a ternary complex on a biosensor surface. The strategy called sandwich hybridization relies on the use of two oligonucleotides: a PPRH hairpin designed to attach to the polypyrimidine sequences of SARS-CoV-2 forming a triplex as the capture probe, and a DNA oligonucleotide designed to be complementary to a region adjacent to the polypyrimidine target site, forming a duplex labeled as the detection probe.

After the TENADA method is validated using a fluorescent DNA microarray, this innovative analytical approach is adapted for use with various biosensor devices, such as thermal and electrochemical lateral flow devices, in addition to fluorescent microarrays.[41]

The first assay is mainly characterized by using a thermal lateral flow (TLF) strip, where samples are allowed to flow after an incubation process. The trimolecular complex is detected and developed by infrared laser-generated heat irradiation.

The second electrochemical device is based on the interaction between the analyte (bound to an enzymatic protein that catalyzes redox reactions) and the triplex-forming probe (PPRH), which is linked to a magnetic nanoparticle (MNP). When hybridization occurs between the analyte and the probe on the MNP surface, the protein becomes attached to the MNP. Thus, after passing through a magnet, the MNP is trapped, and the sensors can detect the presence of the trimolecular complex through specific reactions that generate a measurable electric current (electrochemical detection).

Generally, it can be concluded that the results obtained from the responses of both biosensor models based on triplex formation demonstrate high efficiency in TENADA for viral RNA detection and greater sensitivity compared to the sensor based on duplex formation (Figure 14).[40]



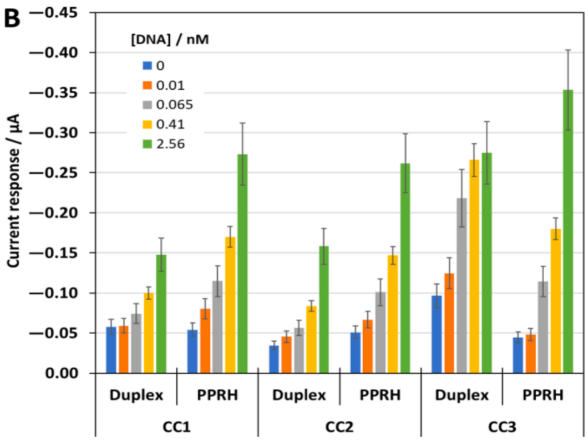


Figure 14: (A) Results obtained with thermal lateral flow system and (B) analytical signal values recorded with three different capture probes (CC1, CC2, CC3) in a duplex and PPRH model (image reprinted with permission from reference *40*)

6.4. TRIPLEX DNA NANOSTRUCTURES IN SENSOR DEVELOPMENT

Triplex DNA nanostructures have become a valuable and indispensable nano-tool in sensing platforms to expand the fields of DNA nanotechnology and aiding in the development of biosensors due to their ability to recognize specific sequences.

Likewise, triplex-forming sequences have found applications in the creation of nanoscale switches due to their pH-responsive properties when combined with other nucleic acid sequences or aptamers.

6.4.1. Aptasensors based on triplex DNA structures

DNA nanostructures have proven to be useful in creating aptasensors for detecting both macromolecules and small molecules. These sensors rely on two key elements: a component for specific recognition and a unit that generates a signal upon target binding.[42]

For this reason, aptasensors utilize aptamers (single-stranded RNA or DNA oligonucleotides with unique intramolecular conformations) as specific ligand recognition elements. These aptamers offer high affinity and selectivity, enabling the integration of various detection strategies. Recognizing the important role of the aptamer selection process, a new SELEX procedure has been developed to preserve the original binding conformation between aptamers and their targets.[43]

Zheng et al. have developed aptasensors utilizing Triple Helix Molecular Switches (THMS) for the detection of thrombin, L-argininamide and ATP. The approach (Figure 15) entails a TFO labeled at both ends with pyrene (blue) as a signal transduction probe (STP), forming an "open" configuration, and a hairpin containing a specific aptameric sequence for the target/analyte (green) as a recognition probe. This aptameric sequence comprises two arm segments linked to the STP via Watson-Crick and Hoogsteen bonds. The interaction of the analyte with the aptamer disrupts the triple helix structure and releases the STP, which assumes a "closed" configuration, resulting in enhanced fluorescence of the pyrenes attached at both 3' and 5' ends of the TFO.[44]

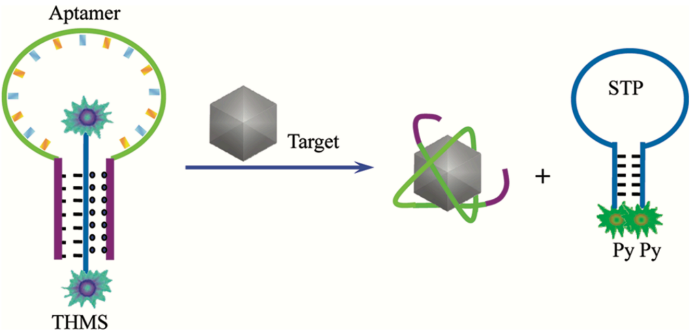


Figure 15: Scheme of signaling aptamer-target binding in THMS, where "-" represent Watson-Crick and "·" represent Hoogsteen base pairings
(image reprinted with permission from *reference 44*)

This design has been employed for the detection of nucleic acids and ATP using Surface-Enhanced Raman Scattering (SERS-THMS), as well as for detecting nucleic acids with signal amplification. These aptasensors exhibit outstanding performance characterized by high sensitivity and specificity, attributed to the strong binding affinity of the aptamers to their ligands. Moreover, the immobilization of triple helix DNA on the surface ensures proper alignment for SERS or electrochemical signal generation.[45]

Generally, aptasensors which are based on THMS provide a practical and widely applicable detection system. Nevertheless, ongoing efforts are directed towards enhancing efficiency and sensitivity, along with optimizing methods to prevent nonspecific hybridizations.

6.4.2. Triplex DNA-based cellular pH sensors

As previously mentioned, some DNA triplex formations are pH-dependent, remaining stable in acidic conditions because cytosine requires protonation. This characteristic has led to the development of diverse DNA nanodevices serving as sensors for both intracellular (pHi) and extracellular (pHe) pH, facilitating applications in *in vivo imaging*.[42]

On one hand, DNA nanodevices sensitive to pHi have been reported, such as biosensors utilizing graphene oxide (GO) and the t-switch nanodevice.

The biosensor utilizing GO, also well-known as fluorescence quencher, serves as a transporter and suppressor of fluorescence by sensing alterations in pH levels. Upon detecting acidic pH environments within living cells, there is a shift between DNA duplex and triplex states, causing the DNA triplex to detach from the GO, thereby emitting fluorescence.[46] The t-switch nanodevice, consisting of three strands (A, B, and C), is based on the DNA duplex-triplex transition, leading to pH-dependent FRET signals (Figure 16). Under high pH conditions, A and B adopt an elongated duplex conformation, separating the two fluorophores preventing FRET in an "open state". Conversely, in acidic conditions, the formation of the C^{+}·G:C triad structure induces a triplex configuration, resulting in a "closed state" that brings the two fluorophores into close proximity and facilitating FRET.[47]

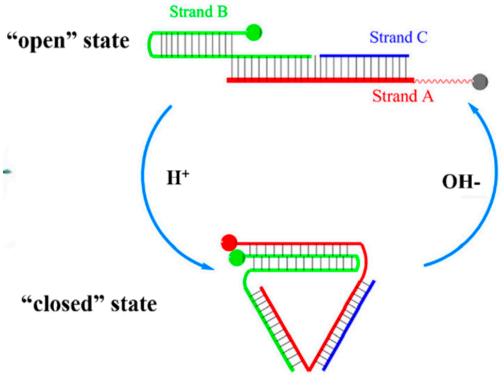


Figure 16: Scheme of t-switch in the “open” state at high pH level and in the “closed” state at low pH level

(image reprinted with permission from *reference 47*)

On the other hand, concerning extracellular pH sensors, Liu et al. designed a DNA nanomachine anchored to the cell surface to identify the distinctive pHe of cancer cells using a pH-sensitive triplex structure, given its tendency to be lower than in healthy cells. When exposed to acidic environments, the triplex structure forms resulting in fluorescence quenching, whereas in basic pH conditions, fluorescence is reinstated.[48]

Despite the potential of these sensors, the difference in fluorescence intensity between pH 5.0 and 8.0 poses limitations in detecting the pHe of cancer cells. This is because the lowest recorded pHe of cancer cells is approximately 6.0, which is insufficient to trigger the duplex-to-triplex transition within living cells.[42]

6.4.3. Sensing protein activity with triplex DNA

A new application associated with triplex-based nanosensors involves the reconfiguration of triplex structures triggered by protein activity.

Human DNA repair enzyme hMGMT is influential in the efficacy of chemotherapy. Researchers Ricci et al. developed a DNA nanoswitch containing O6-methyl-guanine (O6-MeG) nucleobases and a fluorophore/quencher marker. hMGMT removes methyl groups from O6-MeG, allowing triplex formation and altering fluorescence, aiding in the detection of methyltransferase inhibitors.[42]

It has been demonstrated that the presence of methyl groups in guanine bases prevents the formation of Hoogsteen interactions with the adjacent cytosine bases, thus affecting triplex formation. Methylated nanoswitches are efficiently recognized by these enzymes that remove the methyl groups from O6-MeG, enabling triplex formation and causing changes in the FRET signal following repair activity.[49]

Thus, the effect of methylation on triplex formation has been analyzed in three different scenarios. The control triplex nanoswitch lacking O6-MeG (Figure 17A) demonstrates indications of a folded triplex at acidic pH (pH 5.0) and suggests the unfolding of the triplex at basic pH levels (pH 8.5). The presence of one O6-MeG (Figure 17B) significantly disrupts triplex formation, resulting in a FRET signal observed at pH 5.0. Finally, the nanoswitch with two O6-MeG (Figure 17C) is further destabilized, and the pH curve shows no significant change in fluorescence signal at pH 5.0.[49]

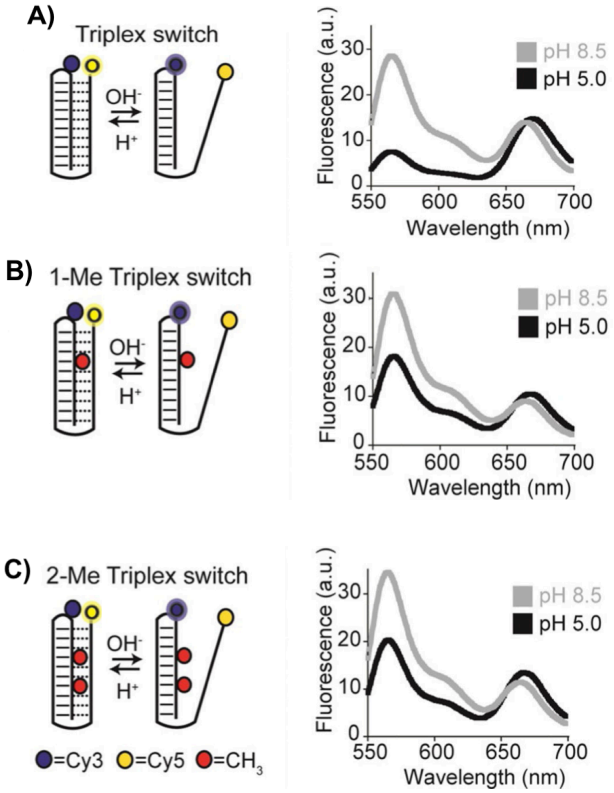


Figure 17: Nanoswitches based on DNA designed to form an intramolecular triplex structure with A) 0 O6-MeG (Triplex), B) 1 O6-MeG (1-Me Triplex) and C) 2 O6-MeG (2-Me Triplex) in the sequence, and their respective fluorescence spectra at pH 5.0 and pH 8.5
(image reprinted with permission from *reference 49*)

On the other hand, a group of researchers has designed a novel application associated with triplex-based sensors to control the binding capacity of antibodies.

Antibodies are proteins produced by the immune system in response to antigens which trigger immune responses, and antibodies specifically engage with these antigens in order to neutralize them. However, challenges arise from false positives and side effects stemming from antigen expression in healthy cells. For this reason, biosensors have been developed to allow reversible control of antibody activity when specific antigens are present.[50]

The strategy uses a pH-sensitive DNA triple helix to control the transition from a robust-bivalent peptide-DNA ligand to a weaker-binding monovalent form. By modifying the DNA triple helix, the designated pH for antibody activation can be adjusted, enabling antibody activation in both alkaline and acidic pH environments.

The results demonstrate that in alkaline pH conditions, the DNA triplex switch destabilizes and generates two weak-binding monovalent ligands that rapidly dissociate, allowing subsequent antibody activation (Figure 18A). In contrast, at acidic pH levels, the formation of the triple helix disrupts the bivalent nature of the ligand, inducing its dissociation into two monovalent peptide-DNA ligands and activating antibody activity function (Figure 18B). [51]

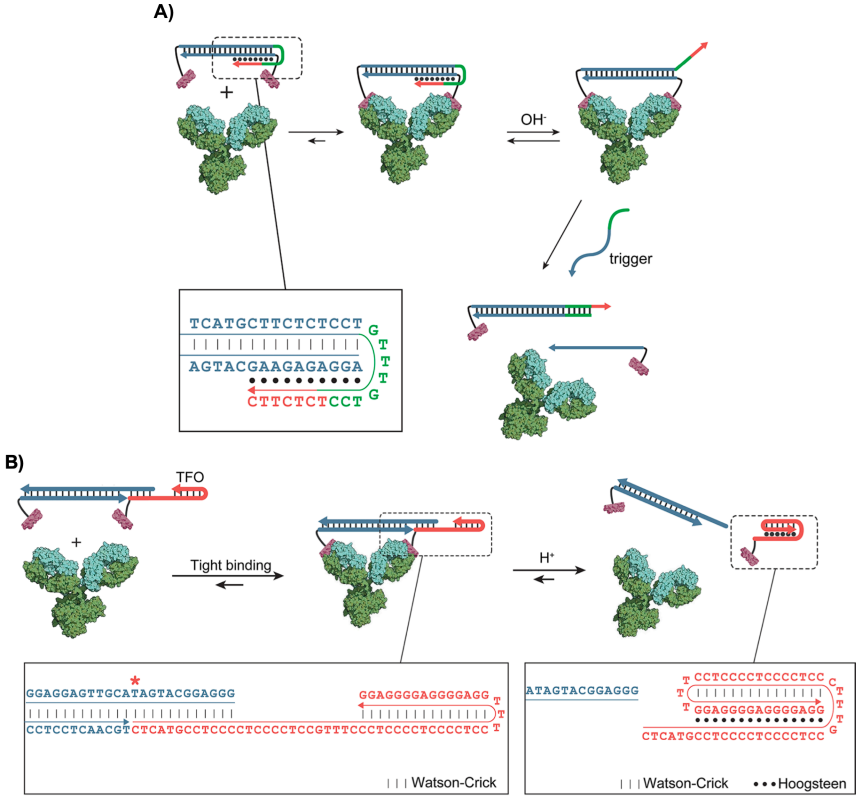


Figure 18: Design of bivalent antibody ligand for antibody activation at (a) alkaline pH and (b) acidic pH

(image reprinted with permission from reference *51*)

Finally, different research groups have also designed biosensors for detecting ions and molecules essential in industrial processes and environmental monitoring. An illustration of this is demonstrated by Lu and Liu, who demonstrate a Cu^{2+}-dependent triplex-based nanodevice. Upon exposure to copper, a DNAzyme (DNA molecule with catalytic activity as a enzyme) identifies and cleaves the DNA substrate, emitting a fluorescent signal. [52]

7. CONCLUSIONS

The research carried out in this bibliographic study allows extracting a series of conclusions that enable the evaluation of the initially proposed objectives:

(1) Firstly, the versatility of the DNA molecule to adopt different structures has been described, including the DNA triple helix. Depending on the orientation of the triplex-forming oligonucleotide, parallel and antiparallel triplexes can be obtained. Parallel triplexes require cytosine protonation to maintain their stability, showing a notable pH dependence, while antiparallel triplexes do not require protonation and therefore are not pH-dependent. Another difference between these structures lies in the hypochromic effect display, which is significantly lower in antiparallel triplexes and therefore presents limitations for their structural study. The different conformations of the DNA triple helix have a significant impact on its stability and consequent functionality, making it useful in various applications. Understanding the unique properties of the DNA triple helix is crucial for its utilization in a variety of technological and scientific fields.

(2) On the other hand, several factors that influence the stability of the triple helix structure have been mentioned throughout the project, including the arrangement of bases, temperature, the presence of certain cations or the pH factor. In addition, series of possible modifications to the nucleobases of the parallel triple helix have been discussed in order to increase its stability, including the incorporation of compounds like 5-methylcytosine, 5-bromouracil, or 5-propynyluracil. Having knowledge and controlling these factors is essential for maintain the structural integrity of the triple helix and developing its potential applications, specifically pH factor which plays a crucial function in stability.

(3) Thirdly, the importance of detecting and characterizing triple helix structure through spectroscopic and electrophoretic techniques has been discussed. Concerning spectroscopic methods, examples such as UV-Vis spectroscopy and circular dichroism are presented in this research, both allowing confirmation of structure formation by detecting alterations in absorbance and conformational changes, respectively. Meanwhile, electrophoretic

techniques, such as gel electrophoresis and capillary electrophoresis, enable the separation and visualization of DNA structures with high resolution, facilitating their identification. Both techniques are useful for analyzing the DNA triple helix. Nevertheless, spectroscopic techniques generally provide valuable data to determine the optimal conditions (pH, presence of cations and temperature) for a favorable interaction between TFO and dsDNA, by turning them into accessible and efficient methods for routine analysis of the DNA triple helix in the laboratory.

(4) The key objective of this work has focused on the analytical and bioanalytical applications provided by the studied DNA triple helix. The incorporation of the DNA triple helix in different detection methods is increasingly being utilized, resulting in a considerable increase in their levels of selectivity. Thanks to the ability of these structures for specific recognition and binding to nucleic acid sequences, advanced biosensors have demonstrated their favorable application in medical diagnostics or in the detection of pathogens in biological samples. For example, the involvement of PPRH oligonucleotides as probes in the TENADA method has allowed the detection of SARS-CoV-2 virus RNA through an alternative method with greater efficiency and speed than the traditional one. Another relevant application of triple DNA nanostructures is the development of biosensors and the creation of nanodevices useful for the reconfiguration of triplex structures in response to protein activity or to control the binding capacity of antibodies.

It is important to emphasize that all the applications offered by the triple helix are currently in stages of research and development.

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9. ACRONYMS

A: Adenine

AODNs: Antisense oligodeoxynucleotides

ATP: Adenosine triphosphate

C: Cytosine

CD: Circular Dichroism spectroscopy

CE: Capillary Electrophoresis

CGE: Gel Capillary Electrophoresis

DNA: Deoxyribonucleic acid

DNAzyme: Deoxyribonucleic acid enzyme

dsDNA: Double-stranded DNA

FRET: Förster Resonance Energy Transfer

G: Guanine

G4FS: Guanine-quadruplex-forming sequences

GO: Graphene oxide

hMGMT: Methylguanine methyltransferase enzyme

HPMC: Hydroxypropyl methylcellulose

miRNAs: MicroRNAs

Me: Methyl

MNP: Magnetic nanoparticle

mtLSU rRNA: Mitochondrial large subunit ribosomal RNA gene

O6-MeG: 06-methyl-guanine

PAGE: Polyacrylamide Gel Electrophoresis

PCR: Polymerase chain reaction

pHe: extracellular pH

pHi: intracellular pH

PPRH: Polypurine Reverse Hoogsteen

PNAs: Peptide Nucleic Acids

Py: Pyrimidine

RNA: Ribonucleic acid

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

SDS: Sodium Dodecyl Sulfate

SELEX: Systematic evolution of ligands by exponential enrichment

SERS: Surface-Enhanced Raman Scattering

ssDNA: single-stranded DNA

SPR: Surface Plasmon Resonance

STP: Signal transduction probe

T: Thymine

TENADA: Triplex Enhanced Nucleic Acid Detection Assay

THMS: Triple Helix Molecular Switches

TLF: Thermal lateral flow

Tm: Melting temperature

tsDNA: triple-stranded DNA

TFO: Triplex-forming oligonucleotide

U: Uracil

UV-Vis: Ultraviolet-Visible spectroscopy