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In Silico Evaluation of Folding and Structural Stability of Aptamers for Application in the Design of a Biosensor for Testosterone Detection†

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Abstract: Currently, dietary supplements contain a wide range of non-specific concentrations of testosterone and/or its synthetic analogs, substances that are not permitted and that pose a risk to public health, which puts into perspective the need to evaluate and regulate the composition of these products. The present project proposes as a control tool, the development of a biosensor using aptamers as bio-recognition elements. The aptamer is a specific sequence of oligonucleotides with can fold into unique three-dimensional structures that interact with the analyte (testosterone and analogs). Integrally, it is proposed that the aptamers are coupled to gold nanoparticles functioning as a census and signal transduction system conducing to a biosensor with high sensitivity and selectivity, and rapid response. In this work, modeling and molecular docking tools were used to evaluate the folding and structural stability of the aptamers. It is essential to carry out the *in-silico* analysis, in a complete way for the bio-recognition system, to evaluate the stability of the proposed aptamers to various variations in the medium, allowing to determine the conditions and adaptations necessary for experimental, design and operation of the biosensor. On the other hand, evaluating the affinity and identifying the type of interactions between aptamer and analyte, allows us to locate the best candidate, for the proposed aptamers. The stability of a set of nine sequences, with proven interaction towards testosterone, was evaluated under different conditions: folding temperature (8.0 °C, 20 °C and 30 °C), [Na^+] (1.0 mm M, 50 mM, and 150 mM) and [Mg^{2+}] (1.0 mm M, 2.0 mM, 3.0 mM, and 4.0 mM), with Mfold web server, RNA Composer and PyMOL. The affinity and molecular interaction assays were carried out between each of the aptamers and three analytes: testosterone, testosterone undecanoate, and androstenedione using Autodock Vina, Chimera, Pymol and Discovery Studio. The results of stability and conformational changes of the aptamers conclude that the aptamers (T6, T5.1, and TESS1) are compatible with the conditions to be run tests and have high affinity for testosterone, whose interactions are constituted mainly by no covalent, and hydrogen bonds.

Keywords: *in silico*; aptamer; biosensor; molecular docking; testosterone-aptamer

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1. Introduction

In México according to "OFFICIAL MEXICAN STANDARD NOM-251-SSA1-2009" we can find out that any anabolic steroids are not permissible substances in food supplements, but it is difficult to control the sale and distribution of these products due to the

easy access of national and imported food substitutes in addition to the growing demand and incorporation of new products to the market; being so that any supplement may or may not contain testosterone and its analogs in a wide range of concentrations, and be the cause of behavioral problems. (Irritability and aggressiveness), personality traits and drifts mood disorders (anxiety, depression) and, also include physical effects such as baldness, cholesteric jaundice, psychotic symptoms, kidney and liver disease, coronary heart disease and cardiovascular events. Following the above, the need to develop detection tools whose use is easier and faster to respond to is addressed, for testosterone and its analogues. The present project is a response to the food supplement lack regulations, principally to the not-allowable substances and their concentrations, much research evaluates the presence and implications of steroids substance like testosterone and their analogs on food supplements, reported by Balasubramanian et al. [1]; Zamil et al. [2]; Jędrejko et al. [3].

According to the above, it addresses the need to develop a detection tool whose use is easier and quick to respond for essential targets: testosterone and its analogs, we propose the development of a biosensor using aptamers as bio-recognition elements, coupled with gold nanoparticles functioning as a census and signal transduction system conducing to a biosensor with high sensitivity and selectivity, as well as rapid response. [4]

In the design of a biosensor, the bio-recognition part plays an important role, so the *in-silico* evaluation of this element is the central part of this writing. Aptamers are single-chain sequences of oligonucleotides with the ability to recognize and with high sensibility for the detection of small molecules, the binding properties of each aptamer not only vary according to the composition of their nucleotide bases but in the same way they are subject to the structural conformation that each aptamer takes and can be modified by the characteristics of the medium. In-silico tools allow to evaluate the stability of the aptamer by maintaining its conformational structure before the variation of temperature and ion plug, likewise, it is possible to identify, the capacity and affinity of union that the aptamers present towards the molecule of interest (testosterone and analogs), it's possible conformational patterns and the type of binding links that occur between the aptamer and testosterone/analogs.

2. Methods

The aptamers considered for the development of the biosensor with proven interaction capacity were subjected to in-silico evaluation for their stability and interaction with testosterone, undecanoate of testosterone, and androstenedione, which are presented in Table 1.

Table 1. Aptamers for the design of the bio-recognition element for the biosensor.

Aptamer	Sequence	Reference
apT5	5′ TAGGGAAGAGAAGGACATATGATTGCG TGGGTAG-GAAGGGGCGGTGTGATCTGAATCGTTCGATTGACTAG-TACATGACCACTTGA 3′	[5]
P4G13	5′ATACCAGCTTATTCAATTGCATCACACACCGA-TACTCACCCGCCTGATTAACATTAGCCCAC-CGCCCACCCCCGCTGCAGATAGTAAGTGCAATCT 3′	[6]
T5	5′ TAGGGAAGAGAAGGACATATGATTGCG TGGGTAG-GAAGGGGCGGTGTGATCTGAATC GTTCGATTGACTAG-TACATGACCACTTGA 3′	[6]
TESS1	5′ CTCTCGGGACGACGGGATGTCCGGGGTA CGGTGGTTGCAGTTCGTCGTCCC 3′	[7]

TESS2	5´ CTCTCGGGACGACCAGGTGCCATTAGCG TCAG-TGTGCTACGATGTCGTCCC 3´	[7]
TESS3	5´ CTCTCGGGACGACGGGTGGTCATTGAG TGGTCTTAGGCAGGTAGTCGTCCC3´	[7]
T4	5´ AGGGAAGAGAAGGACATATGATCCTTG CCATGTT-GGGACATCGTTTTACGGGCCTCTT CAGAATTACTGTTT-GACTAGTACATGACCA CTTGAGG 3′	[8]
T5.1	5´ TAGGGAAGAGAAGGACATATGATGTGCC GTGAATACAGGCCCTTCTCCGCTCCGCGTTCCGATTT-GACTAGTACATGACCACTTGAGG 3´	[8]
T6	5´ TAGGGAAGAGAAGGACATATGATGTGC CGTGAATACAGGCCCTTCTCCGCTCCGCGTTCCGCTTT-GACTAGTACATGACCACTTGA 3´	[8]

For the structural evaluation of the nine aptamers to different conditions, and each tertiary structure modeled, their affinity towards testosterone, testosterone undecanoate, and androstenedione was evaluated.

2.1. Prediction of the Secondary Structure

A library of its secondary structures was generated, in CT format, based on a model of free energy minimization, applying the Mfold web server [9], where the temperature conditions at 8.0 °C, 20 °C and 30 °C were modified individually for each aptamer; the ion plug conditions, with sodium [Na^+] concentrations at 1.0 mm M, 50 mM, and 150 mM and with magnesium concentrations [Mg^{2+}] at 1.0 mM, 2.0 mM, 3.0 mM, and 4.0 mM.

2.2. Construction and Optimization of the Tertiary Structure

They modeled the tertiary structures for each secondary structure generated, using the RNA Composer web server [10], where the CT format was converted into a dot-bracket format; and on the same server the tertiary structure was obtained in .pdb format (Protein Databank) File) to be displayed later in the PyMOL software.

2.3. Molecular Docking.

It obtained the tertiary structures of the targets with the help of the PubChem web server in ".sdf" format (Simulation Description Format); for testosterone (CID: 6013), testosterone undecanoate (CID: 65157), and androstenedione (CID: 6128) for each target the extension was modified to ".pdb" by the Open Babel software.

The tertiary structures after using the RNA Composer web server are equated as RNA sequences, so, the 3DNA-Nucleic Acid Structures web server was used to pass the structures to DNA, later the tertiary structure was optimized with the Avogadro software.

The molecular docking was carried out individually for each tertiary structure modeled of aptamer and for each target, using the Autodock Vina tool in the Chimera software, [11], the generated files were modified by Open Babel software (".pdbqt" to ".pdb" format) to visualize the interactions and links of union between aptamer and target, using Pymol software and Discovery Studio.

3. Results and Discussions

In the *in-silico* evaluation of the folding and structural stability of the aptamers, the value of ΔG is obtained, in the generation of secondary structures, where it is considered thermodynamically stable when its value is negative and whose numerical magnitude will denote equivalently the magnitude of the stability. Consequently, the folding pattern can be visualized as conditions change, presenting more or less loops, forks, protrusions, and pseudo-knots in the spliced tertiary structure models.

Below it presented the ΔG value in Table 2 is distinguished by different conditions to which the modeling of each aptamer was subjected, and then the structures tertiated by aptamer are observed (Figure 1).

Table 2. ΔG obtained through the modeling of the secondary structure of aptamers varying the conditions: for different temperatures in Celsius degrees (T °C), different concentrations of sodium ion in millimolar ([Na^+] mM), and different concentrations of magnesium ion in millimolar. ([Mg^{2+}] mM).

APTÁMERO	T °C	Δ*G* (kcal/mol)	[Na+] mM	Δ*G* (kcal/mol)	[Mg2+] mM	Δ*G* (kcal/mol)
apT5	**8**	-1.31	**1.0**	0.45	**1.0**	-2.26
	20	-0.45	**50**	-1.58	**2.0**	-2.64
	30	-1.2	**150**	-2.64	**3.0**	-2.84
					4.0	-3.07
P4G13	**8**	-3.46	**1.0**	-0.69	**1.0**	-4.40
	20	-0.69	**50**	-3.41	**2.0**	-4.89
	30	0.43	**150**	-4.90	**3.0**	-5.15
					4.0	-5.36
T5	**8**	-1.13	**1.0**	0.45	**1.0**	-2.26
	20	-0.45	**50**	-1.58	**2.0**	-2.64
	30	-	**150**	-2.64	**3.0**	-2.84
					4.0	-3.07
TESS1	**8**	-10.37	**1.0**	-6.12	**1.0**	-12.67
	20	-6.12	**50**	-11.37	**2.0**	-13.31
	30	-3.69	**150**	-13.32	**3.0**	-13.67
					4.0	-13.97
TESS2	**8**	-6.43	**1.0**	-3.87	**1.0**	-8.64
	20	-3.87	**50**	-7.27	**2.0**	-9.32
	30	-1.77	**150**	-9.33	**3.0**	-9.68
					4.0	-9.96
TESS3	**8**	-6.95	**1.0**	-4.30	**1.0**	-8.05
	20	-4.30	**50**	-7.47	**2.0**	-8.35
	30	-3.20	**150**	-8.35	**3.0**	-8.50
					4.0	-8.62
T4	**8**	-7.29	**1.0**	-2.47	**1.0**	-9.36
	20	-2.47	**50**	-7.77	**2.0**	-10.18
	30	-0.13	**150**	-30.83	**3.0**	-10.60
					4.0	-10.97
T5.1	**8**	-7.73	**1.0**	-2.40	**1.0**	-9.28
	20	-2.40	**50**	-8.15	**2.0**	-9.95
	30	-1.13	**150**	-9.95	**3.0**	-10.31
					4.0	-10.64
T6	**8**	-8.12	**1.0**	-2.83	**1.0**	-10.02
	20	-2.83	**50**	-8.75	**2.0**	-10.78
	30	-1.18	**150**	-10.75	**3.0**	-11.19
					4.0	-11.55

According to the values presented in Table 1 above, for most cases, except for the aptamer apT5, the aptamers destabilize as the temperature increases, because at high temperatures the bonds generated between nucleotide bases tend to separate.

At various concentrations of [Na^+], the ΔG value (kcal/mol) decreases as the sodium concentration increases, in response to the capacity of sodium as a stabilizing cation for nucleic acids by reducing the solubility that surrounds the sequence allowing it to generate a greater number of interactions with each other.

By evaluating the aptamers in concentrations of [Mg^{2+}], the ΔG value (kcal/mol) decreases as the concentration of magnesium increases, that is, the higher the concentration of magnesium is assumed to be thermodynamically more stable, this phenomenon is because the magnesium ion has the characteristic of joining with the deoxyribose's and forming complexes.

The relationship between Table 2 and Figure 1 scheme (a), also visualized in the appendix, Figure A1, agrees with the behavior described, in which the most compact tertiary structures, are those that present conformations with a greater number of loops and forks. It is observed, in Figure 1 (a), that some of the aptamers have almost zero structural changes such as TESS3 and TESS2 contrary to the aptamers P4G13, T5, apT5 that modeled at 8 °C and 20 °C have significant changes in their structure, which decrease the possibility of adapting to the conditions of execution of the tests. The aptamers T4, T6, T5.1, and TESS1 retain structure between the models generated at 8 °C and 20 °C, in addition to being the aptamers with the highest negative sign values of ΔG, above ΔG = -10.00 (Table 2), postulating as thermally stable aptamers.

According to Table 2 and Figure 1 (b), also visualized in the appendix Figure A2, the aptamer TESS3 retains its structure at any concentration. The partially stable aptamers T6 and P4G13 have stability between concentrations 1.0 mM to 50 mM; TESS1 and TESS2 between 50 mM and 150 mM. For aptamers apT5, T4, T5.1, and T5, they present noticeable changes in each concentration.

According to Table 2 and Figure 2 (c), also visualized in the appendix Figure A3, the molecular models present slight structural changes, however, there are aptamers whose conformational changes are more noticeable as apT5 and T5. For aptamers T4, TESS1, T6, T5.1, and TESS2 present a single structural change, while P4G13 and TESS3, had no conformational changes.

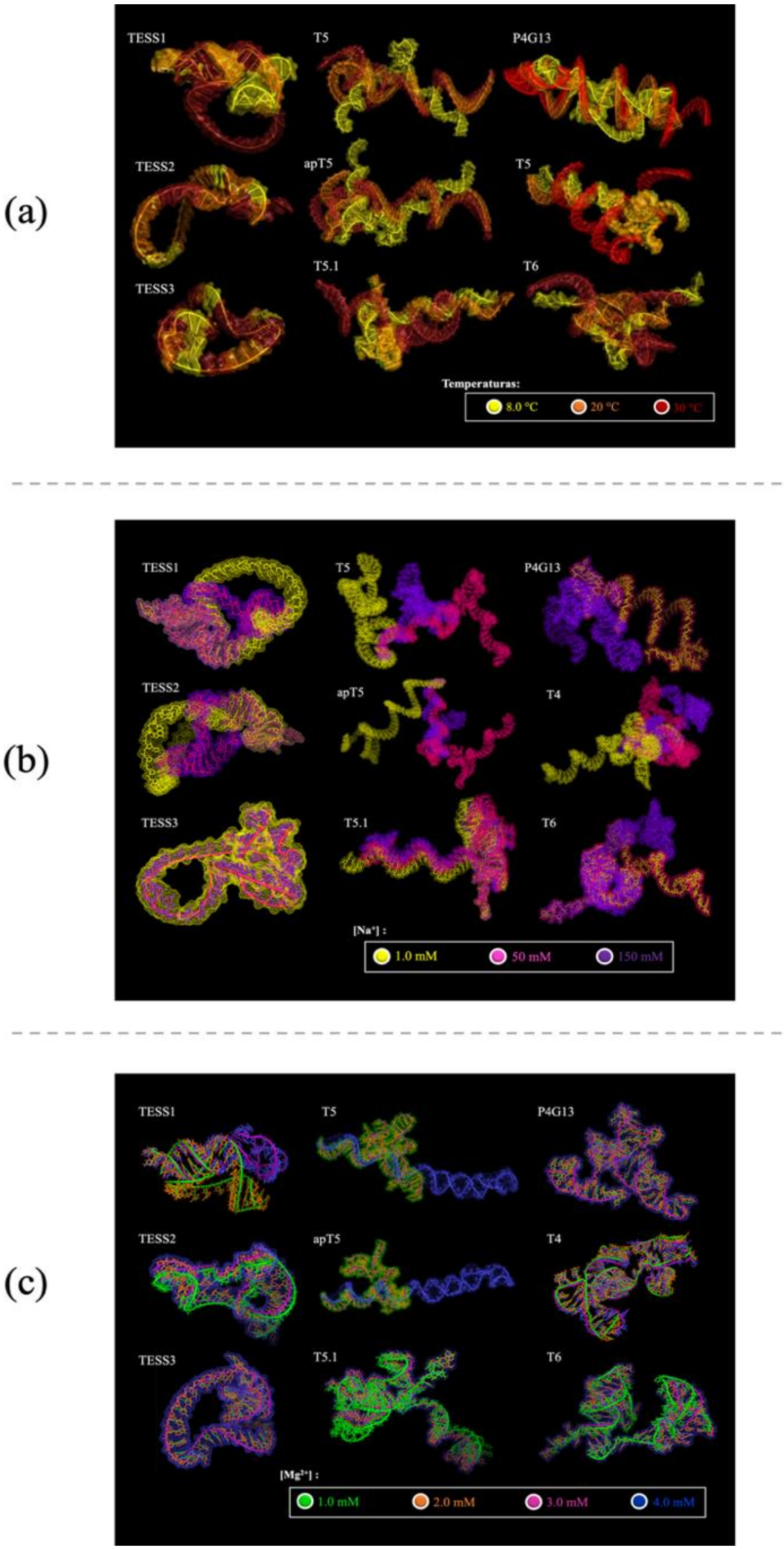


Figure 1. Tertiary structures of the nine aptamers modeled under different conditions (a) Different temperatures with color correspondence at 8.0 °C yellow, 20 °C orange, and 30 °C red; (b) Different concentrations of [Mg^{2+}] with color correspondence to 1.0 mM green, 2.0 mM orange, 3.0 mM rosy color and 4.0 mM blue; (c) Different concentrations of [Na^+] with color correspondence at 1.0 mM yellow, 50 mM rosy color and 150 mM purple.

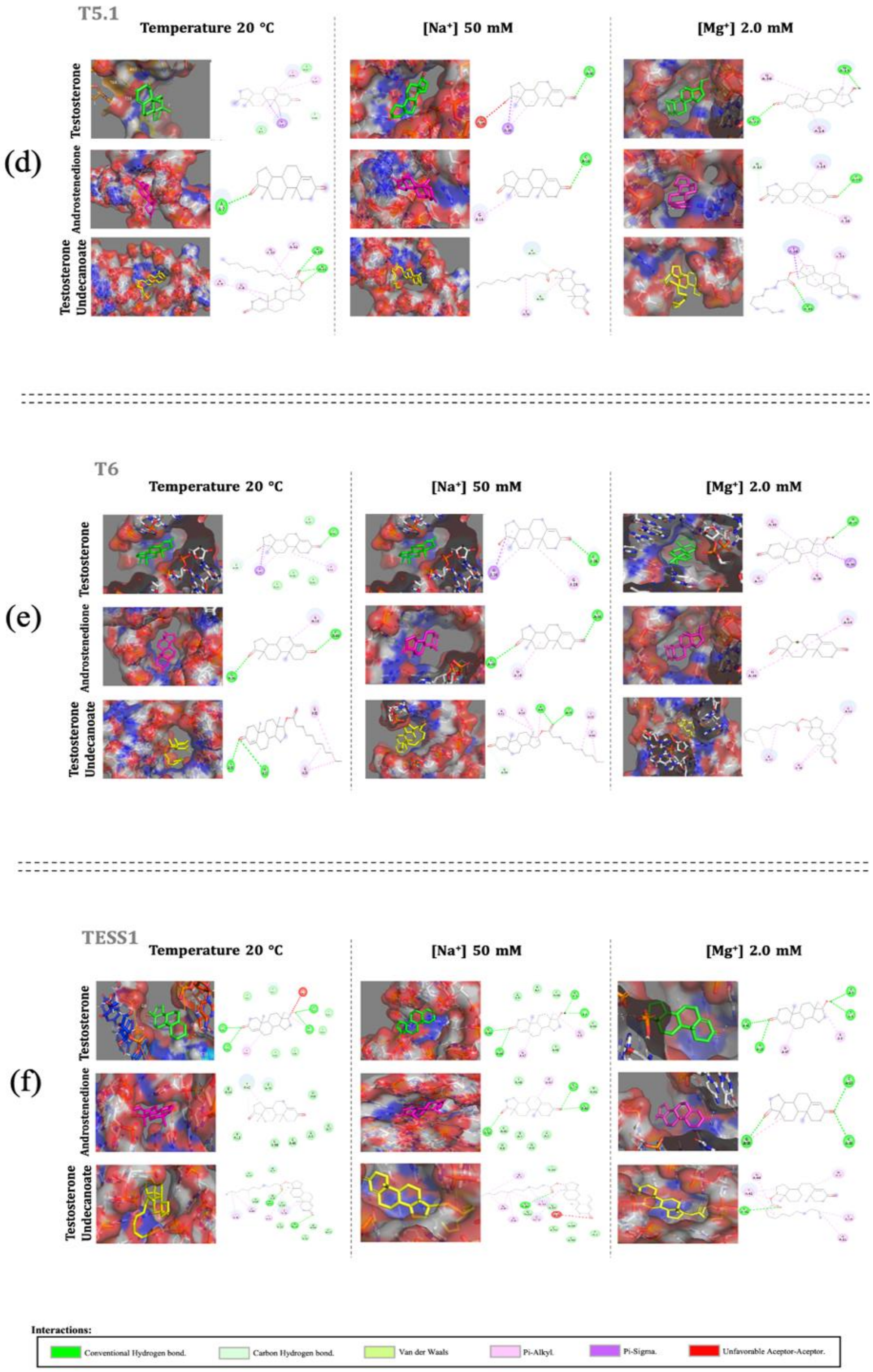


Figure 2. Molecular docking for (d) T5.1, (e) T6, and (f) TESS1. Using Pymol images on 3D structure visualization and Discovery studio for the images on 2D structures to visualize all their interaction, aptamer-target.

Relating to the above results, we select three aptamers with structural stability, a higher value of ΔG, and less configurational change on the different conditions. To start with molecular docking, we focus on the selected aptamers (T5.1, T6, TESS1) and the average conditions of experimental work, that we infer to work in the future: temperature at 20 °C, sodium ion concentration at 50 mM, and magnesium ion concentration at 2.0 mM.

For convenience, each scheme (d), (e), and (f) are split into an appendix section for better visualization.

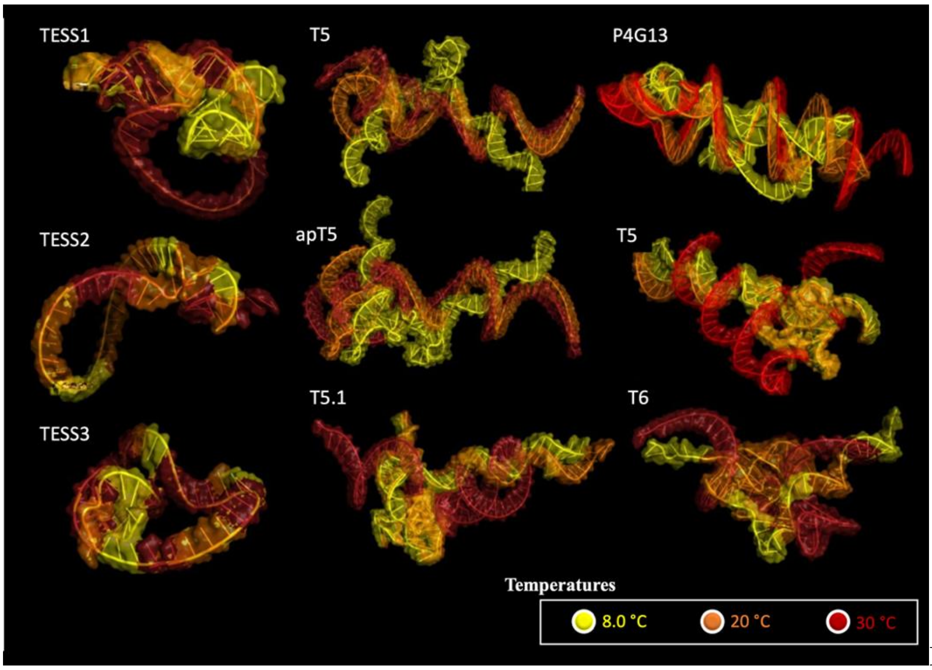
According to Figure 2, the conformations of the aptamers result in a kind of cavity or pseudo-cavity where the target is already testosterone, androstenedione, or testosterone undecanoate, is located for its union, these junctions are mostly made up of conventional hydrogen bonds, carbon-hydrogen bond, pi-alkyl, and pi-sigma bonds.

The aptamers T5.1 and T6, have more links pi-Alkyl (Pi-Alkyl, pink) which, despite being considered weak intermolecular forces, are interactions modulating affinity and selectivity between protein-ligand complexes, a scheme very similar to our aptamer-target, as well as the interaction Pi-Sigma (purple). Conventional Hydrogen bonds are another type of interaction that also occurs with high incidence, these links are related to molecular recognition to be dynamic bonds and minimize the competitiveness with water molecules, finally around carbon - hydrogen interactions are shown that are distributed around the aptamer, due to the shape of a cavity that "wraps" the target. TESS1 presents Conventional Hydrogen bonds, Van der Waals forces in greater quantity than other types of interactions, and two situations an unfavorable interaction acceptor-acceptor, which is directly related to the conformational structure it takes.

4. Conclusions.

It was discarded aptamers that did not possess thermal stability as the aptamer P4G13 and/or stability on ionic plug as the aptamers T5, apT5, and T4 within the compatible conditions to run tests, working molecular docking only with the aptamers T6, T5.1 and TESS1, where characteristic links are observed to the junction aptamer-target in greater quantity for the aptamers T6 and T5.1 which indicates that the generation of tertiary structures to different conditions, directly modifies the type and number of interactions, seen through molecular docking; which reaffirms the importance of in-silico evaluations to provide a suitable as the appropriate biorecognition site for a biosensor.

5. Appendix



For supplement visual material we present the subtract of each scheme from the figures presented in the result and discussion section.

Figure A1. From Figure 1, scheme (a). Different temperatures with color correspondence at 8.0 °C yellow, 20 °C orange, and 30 °C red.

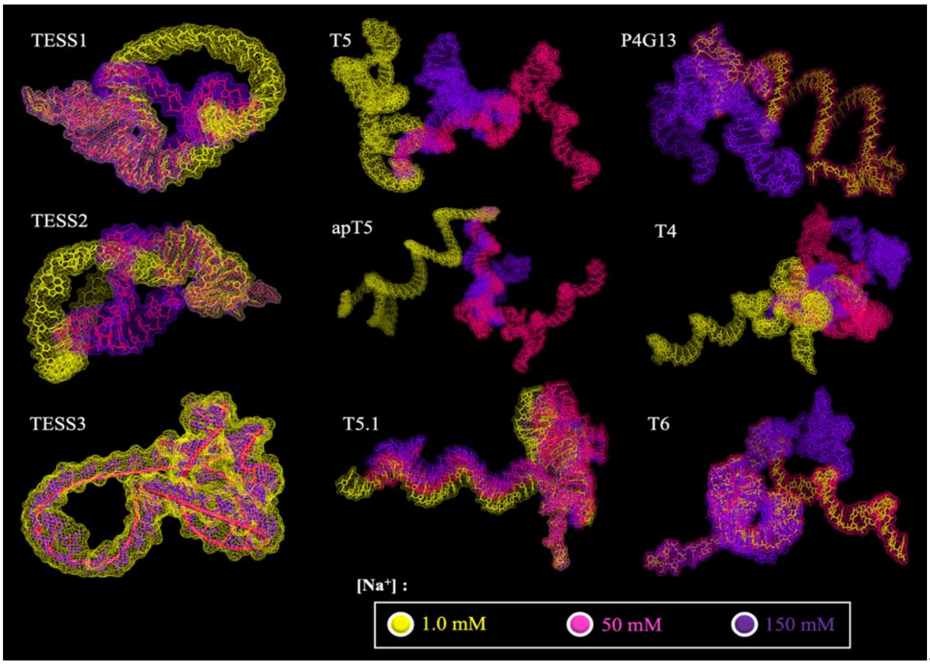


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Figure A2. From Figure 1, scheme (b). Different concentrations of [Na+] with color correspondence at 1.0 mM yellow, 50 mM rosy color and 150 mM purple.

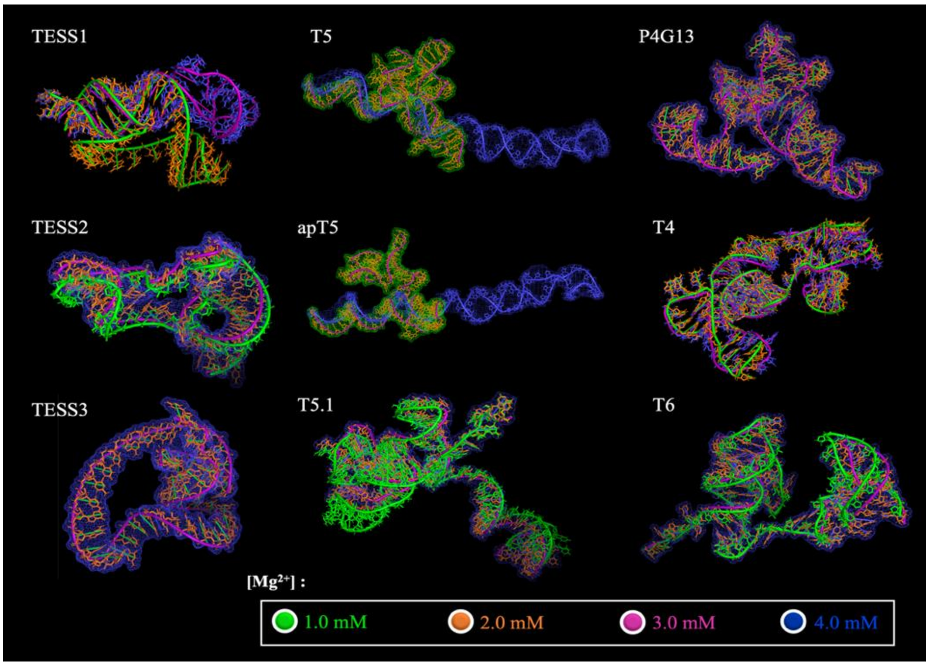

Figure A3. From Figure 1, scheme (c). Different concentrations of [Mg2+] with color correspondence to 1.0 mM green, 2.0 mM orange, 3.0 mM rosy color and 4.0 mM blue.

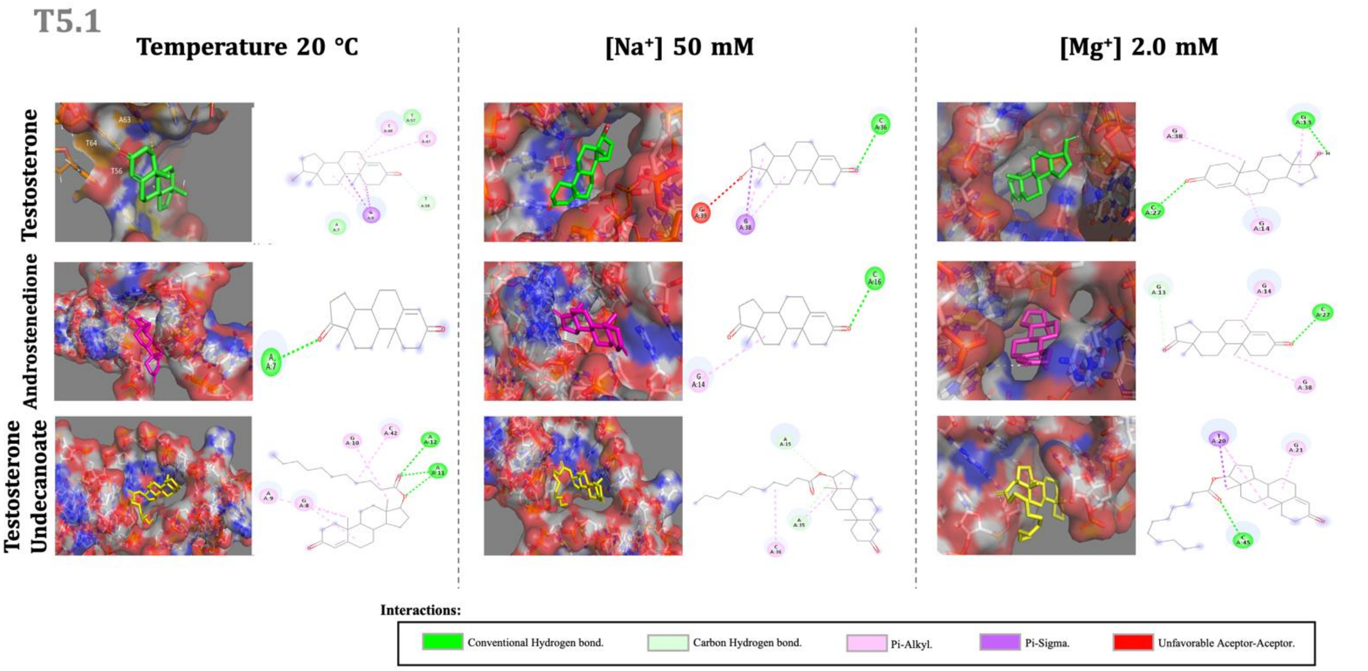

Figure A4. From Figure 2, scheme (d). Molecular docking for T5.1. Using Pymol images on 3D structure visualization and Discovery studio for the images on 2D structures to visualize all their interaction, aptamer-target.

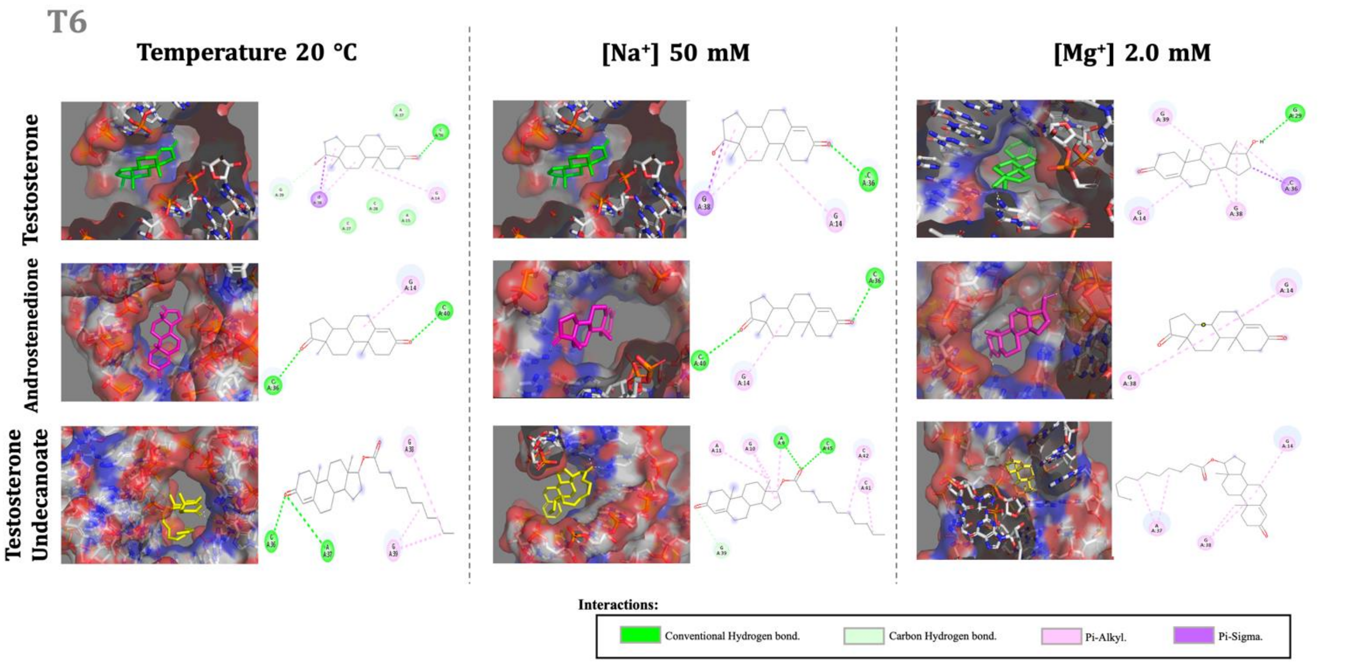

Figure A5. From Figure 2, scheme (e). Molecular docking for T6. Using Pymol images on 3D structure visualization and Discovery studio for the images on 2D structures to visualize all their interaction, aptamer-target.

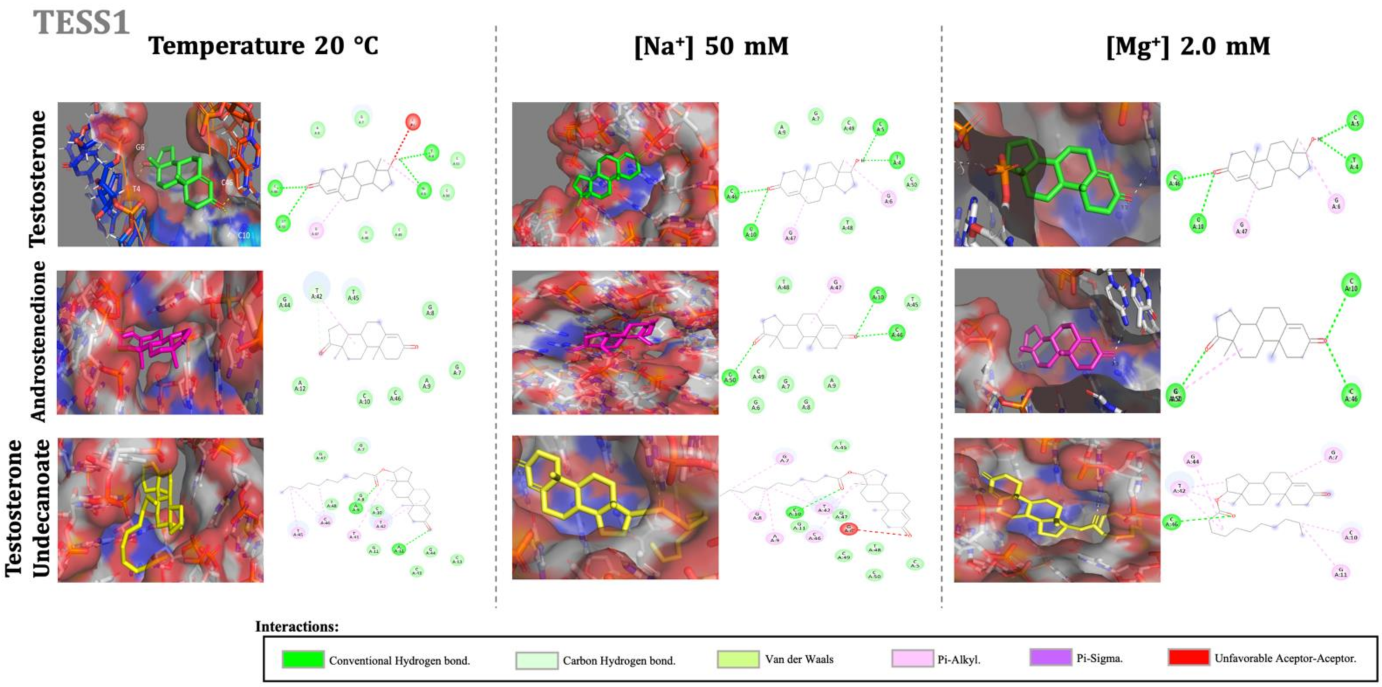

Figure A6. From Figure 2, scheme (f). Molecular docking for TESS1. Using Pymol images on 3D structure visualization and Discovery studio for the images on 2D structures to visualize all their interaction, aptamer-target.

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