Babol University Of Medical Sciences

IJMCM, Spring 2025, VOL 14, NO 2

International Journal of Molecular and Cellular Medicine

Journal homepage: www.ijmcmed.org

ORIGINAL ARTICLE

Innovative Aptamer-HRP Conjugation for Cardiac Troponin I Detection: A Novel ELASA Approach for AMI Diagnostics

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ARTICLE INFO

Received: 2024/11/26
Revised: 2025/02/01
Accepted: 2025/03/15
DOI: 10.22088/IJMCM.BUMS.14.2.705

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ABSTRACT

Acute myocardial infarction (AMI), a major global cause of mortality, is diagnosed using cardiac troponin I (cTnI). Antibody-based assays face challenges, prompting the exploration of aptamers. This study develops an aptamer-HRP probe and ELASA for improved cTnI detection.

Aptamer-based enzyme-linked aptamer assays (ELASA) were developed to detect cTnI using Tro4 and Tro6 aptamers. Molecular docking was performed via the HDOCK web server to confirm aptamer binding affinity to cTnI. Tro4 was biotinylated for use as a capture probe, while Tro6 was conjugated to HRP through sulfo-SMCC crosslinking, followed by size exclusion chromatography and purification. Direct and sandwich ELASA assays were performed using streptavidin-coated plates and clinical serum samples from AMI and non-AMI patients. Data were analyzed using GraphPad Prism10 and SPSS software.

Molecular docking confirmed the high binding affinity of Tro4 and Tro6 aptamers to cTnI, with significant interaction energies. Direct ELASA verified aptamer binding, and optimal concentrations were determined as 10µM for Tro4 and 5µM for Tro6. A sandwich ELASA using paired aptamers achieved improved sensitivity and specificity for cTnI detection. The assay displayed a linear response between 0.1–22 ng.mL cTnI (R^2=0.94), with a limit of detection (LOD) of 0.10ng.mL. When tested on patient serum samples, results correlated with a commercial antibody-based ELASA kit.

This study successfully developed a highly sensitive and specific sandwich ELASA for cTnI detection, utilizing the optimal aptamers Tro4 and Tro6. The results demonstrated excellent sensitivity, specificity, and potential clinical applicability, offering a promising alternative to antibody-based assays.

Keywords: Cardiac Troponin I (cTnI), Aptamer-based ELASA, Acute myocardial infarction (AMI), Biomarker detection

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[DOI: 10.22088/IJMCM.BUMS.14.2.705]

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Introduction

Cardiovascular diseases (CVDs) are a leading cause of mortality worldwide, with acute myocardial infarction (AMI) being the most critical contributor to CVD-related deaths (1). Early and accurate diagnosis of AMI is crucial for effective clinical intervention. While electrocardiography (ECG) is widely used, its sensitivity is often insufficient, necessitating additional data from circulating cardiac biomarkers (2).

During AMI, ischemia or hypoxia damages cardiac myocytes, leading to the release of biomarkers such as myoglobin, creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), and cardiac troponins (cTnI and cTnT) into the bloodstream (3). Among these, cardiac troponin I (cTnI) is recognized as the gold standard biomarker for detecting myocardial damage due to its high specificity and sensitivity (4). Detectable levels of cTnI appear within 4–6 hours after myocardial injury, making it a reliable indicator (5). Immunoassays such as ELISA, chemiluminescence, and rapid diagnostic tests are commonly employed to measure cTnI levels (6).

Hese methods typically rely on antibodies for target detection, which, while effective, present challenges such as high production costs, stability concerns, and batch-to-batch variability (7, 8). Aptamers, single-stranded nucleic acid sequences, have emerged as promising alternatives to antibodies. They offer numerous advantages, including lower production costs, high stability, ease of modification, and the ability to target a broad range of molecules, including small and non-immunogenic targets (9-12). In 2015, Changill Ban et al. developed specific cTnI-targeting aptamers using the SELEX method, identifying two aptamers, Tro4 and Tro6, with high binding affinity (low dissociation constants). These aptamers have since been explored in various molecular diagnostic applications (13-16).

This study introduces a novel enzyme-linked aptamer-based sandwich assay (ELASA) using Tro4 and Tro6 to detect cTnI with high sensitivity and specificity. By leveraging aptamers instead of antibodies, the developed ELASA method overcomes traditional immunoassay limitations and offers a cost-effective, stable, and scalable platform for cTnI detection. This novel approach has the potential for clinical application in AMI diagnosis.

Methods

Chemical reagents

Horseradish peroxidase (HRP) and Sephadex G-25 were purchased from Sigma-Aldrich (USA). Sulfo-SMCC linker was obtained from Thermo Fisher (USA). Streptavidin-coated plates were purchased from Pishtaz Teb (Iran). A cTnI ELISA kit was sourced from Monobind (USA). HRP-streptavidin was obtained from Arghavan Teb Kavian (Iran), and cTnI from Vidas (France). Reagents including Tris, $MgCl_2$, NaCl, KCl, $Na_2\ HPO_4$, $KH_2\ PO_4$, and Tween 20 were procured from Merck (Germany).

The TMB substrate and stop solution were purchased from Monobind (USA), bovine serum albumin (BSA) was sourced from DNA Biotech (Iran), and a 30 kDa Amicon filter from Jet Biofil (China). HPLC-purified DNA aptamer sequences were purchased from Metabion (Germany).The sequences of the aptamers used in this study were:

Tro4:5'_biotin_CGTGCAGTACGCCAACCTTTCTCATG CGCTGCCCCTCTTA. Tro6:5'_thiol_C6_CGCATGCCA AACGTTGCCTCATAGTTCCCTCCCCGTGTCC.

Molecular docking of aptamers

The TRO4 and TRO6 aptamer sequences used in this study were adapted from Changil Ban et al.'s study, and molecular docking was used solely for validating the accuracy of these published sequences. Molecular docking evaluated the interaction between aptamers and the cTnI protein. The HDOCK webserver was used to predict structural interactions and binding affinities. Docking scores were used to determine the most energetically favorable binding modes. Docking scores obtained from the simulation represent the estimated free energy of binding (ΔG). A more negative score implies a higher predicted affinity between the aptamer and cardiac troponin I. These scores were used as a screening tool to identify candidates for further in vitro validation. The docking scores were compared with experimental binding affinities obtained from ELASA assays to assess their predictive accuracy.

HRP conjugation with aptamer

Initially, 10 mg HRP enzyme was dissolved in 1 mL of 0.1 M sodium phosphate buffer with 0.15 M NaCl, pH 7.2.

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Then, 2 mg of sulfo-SMCC linker was added and mixed gently to dissolve. It was incubated at room temperature for 1 hour with periodic mixing. The activated HRP was purified using size exclusion chromatography. Preparing of size exclusion chromatography was performed by packing swollen Sephadex G-25 in a column. Then, activated HRP was injected into the column and chromatography was performed using 0.1 M sodium phosphate, 0.15 M NaCl, and pH 7.2.

As the HRP molecules elute, the eluent was monitored visually for the characteristic brown color of HRP. During purification process, 5 ml fractions were collected and the peak fractions containing HRP were pooled. Pooled fractions were analyzed with spectrophotometry to confirm the activation of HRP. Subsequently, 100µL of activated HRP was incubated with 100 µL thiolated aptamer (100mM) over night at 4°C. Then the thiolated group on the 5' end of the aptamer crosslinked to the maleimide group of sulfo-SMCC. Ultimately, conjugated aptamer was purified by using a 30 kDa amicon filter at 4°C with 3000×g.

After first centrifugation step, 10 ml phosphate buffer was used for washing and the centrifuge was repeated. Two washing steps were performed to increase conjugate purification. The purified aptamer-HRP conjugate was collected from the filter. Confirmation of conjugation was done by evaluating the performance of the conjugated aptamer through direct ELASA assay.

Aptamer-based direct ELASA assay

Both aptamers were utilized for direct enzyme-linked aptamer assay to validate the findings of the in silico phase to in vitro condition. 100µL of cTnI with 380 ng.l concentration was coated in microplate wells at 4℃ overnight. The coated wells were washed five times with PBS-Tween 0.05% and then blocked with 1% BSA for 1h at room temperature. In each well, 100µL of biotinylated aptamer with different concentrations (0.5, 1, 10) was added and incubated for 1h at room temperature. Then wells were washed three times with PBS-Tween 1% and 100µL TMB chromogenic reagent added. After 20 min incubation at room temperature, stop solution was added, and optical density was read at 450 and 630nm. This protocol was also used for different concentrations (1, 2, 5) of HRP-labeled aptamer to determine whether the aptamer binds to the cTnI.

Aptamer-based sandwich ELASA assay

We set up a sandwich assay for the detection of cTnI in 96-well microplates. Sandwich ELASA was developed with a biotinylated aptamer (Tro4) as a capture probe and an HRP-labeled aptamer (Tro6) as a detection probe. Kd of Tro4 and Tro6 is 317 and 270 pM respectively, hence, these two aptamer sequenses have high binding affinity to cTnI protein. Tro4 was dissolved in 20 mM tris buffer, 1 mM mgcl2, and 1 mM EDTA. Tro6 was dissolved in 1x PBS and 50mM mgcl2. First, 5 µL of biotinylated aptamer (100µM) was added to 100 µL serum samples.cTnI antigen, and 100 µL buffer tris 10 mM includes 10 mM mgcl2, 300 mM NaCl, 50 mM Kcl which was adopted from Hyoban Lee et al. and incubated in room temperature for 30 minutes(14).

The streptavidin-coated wells were incubated with the biotinylated aptamer and serum samples for 60 minutes at room temperature. Non-binding molecules were washed away with 300 µL of PBS-Tween 0.05% three times, then HRP-labeled aptamer (10µM) in 100µL buffer tris 10 mM includes 10 mM mgcl2, 300 mM NaCl, 50 mM Kcl was applied to each well for 60 minutes. The wells were washed three times and chromogenic reagents were introduced. After 20 minutes, chromogenic reactions were terminated with the stop solution and the absorbance at 450 and 630nm was collected (Fgure 1).

Collection of clinical serum samples

This study was based on the ethics approval number 1401.483REC.MSP.SBMU.IR from Shahid Beheshti University of Medical Sciences, and all research ethics principles were strictly adhered to during the research. Serum samples were collected from Modares Hospital, Shahid Beheshti University of Medical Sciences, and stored at −80°C. A total of 52 samples (27 from AMI patients and 25 from non-AMI individuals) were analyzed.

Clinical serum samples used in this study were leftover samples that had already been collected as part of routine procedures in the laboratory. These samples were anonymized, and no patient-specific identifiers were used in the research. Therefore, informed consent was not required as per the ethical guidelines of the institution. Sample collection criteria included patients aged 30 years and older, with confirmation of acute myocardial infarction (AMI) in these patients by a cardiologist. The aptamer-based sandwich ELASA

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results were compared with a commercial antibody-based ELISA kit (Monobind, USA).

Statistical analysis

The HDOCK webserver (http: hdock.phys.hust.edu.cn.) was used to predict the tertiary structure of the aptamers. To analyze the relationship between two variables, a Pearson correlation test was applied. The coefficient of determination (R^2) was calculated to quantify the strength of this linear relationship. The Pearson correlation coefficient (r) ranges from -1 to +1, with values closer to ±1 indicating a stronger correlation. R^2 values range from 0 to 1, with values closer to 1 indicating a stronger and more predictive relationship. Statistical significance was considered for P values<0.05. Data analysis and visualization were performed using GraphPad Prism version 10 and SPSS version 26.

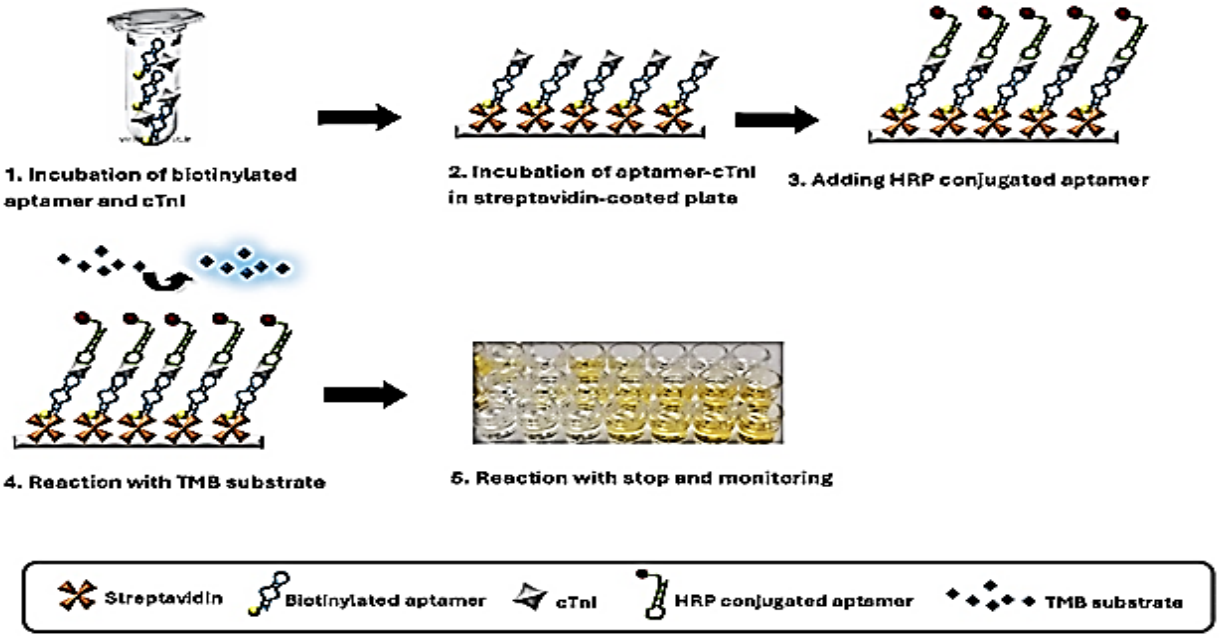

Figure 1. Schematic view of sandwich ELISA using pair aptamer. (1) First, the biotinylated aptamer was incubated with cTnI and Tris buffer at room temperature. (2) Then aptamer-cTnI was coated on streptavidin plates. (3) Tro6 aptamer conjugated with HRP enzyme was added to the wells. (4) After adding TMB and incubation in the dark, (5) stop solution was added and monitored at 450 and 630nm.

Results

Molecular docking of aptamers

Molecular docking analysis conducted using the HDOCK web server demonstrated that both Tro4 and Tro6 aptamer sequences exhibited strong binding potential to the cTnI protein. The docking scores indicated significant interaction energies, suggesting a high likelihood of aptamer-cTnI binding. Confidence scores further supported the aptamers' binding specificity and an indication of the reliability or accuracy of the predicted binding site. The docking scores of the aptamers ranged from [-244 to -198 and -209 to -182], with aptamer TRO4 and TRO6 demonstrating the most favorable binding energy (ΔG=-5.9 and ΔG=-1.3). Subsequent ELASA validation confirmed that aptamer TRO4 and TRO6 exhibited the highest binding affinity to cardiac troponin I, consistent with its docking score. The HDOCK algorithm assumes rigid structures for both the aptamers and protein during docking simulations. However, it indirectly accounts for the flexibility of these molecules by generating multiple conformations of the ligand during the docking process. The flexibility is reflected in the ligand root mean square deviation (rmsd), which measures the deviation of the predicted binding pose from the reference structure of the ligand. A high ligand rmsd, as observed in our models, suggests that the predicted structure for cTnI deviates from its crystallographic structure, indicating

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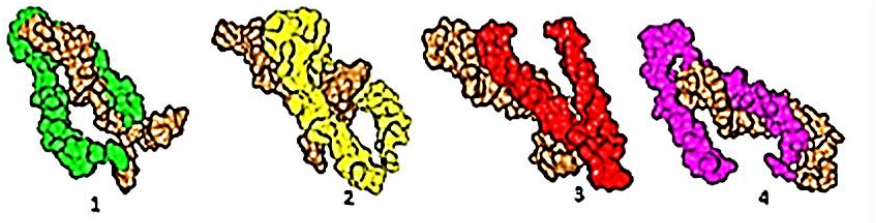
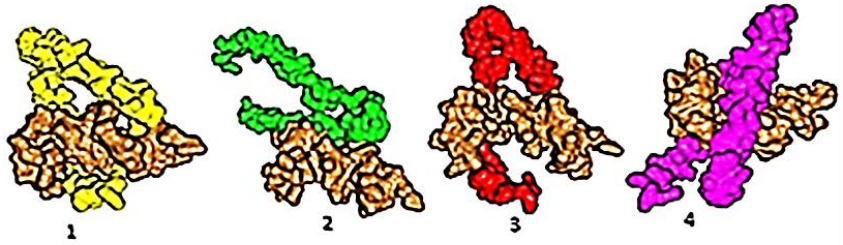
potential conformational flexibility in both the aptamers and the protein. Detailed analysis of all possible structural interaction modes revealed distinct and energetically favorable aptamer-protein conformations, highlighting their potential for targeted binding. These findings underscore the feasibility of employing Tro4 and Tro6 aptamers in detection assays for cTnI (Figure 2).



Rank	1	2	3	4
Docking Score	-244.47	-225.35	-215.71	-214.34
Confidence Score	0.8687	0.8186	0.7882	0.7836
Ligand rmsd (Å)	167.67	146.19	162.97	148.64



Rank	1	2	3	4
Docking Score	-209.39	-205.69	-195.27	-191.24
Confidence Score	0.7663	0.7528	0.7121	0.6953
Ligand rmsd (Å)	312.96	316.95	311.17	320.13

Figure2. Four modes of Tro4 and Tro6 molecular docking with Docking score and confidence score from HDOCK web server. The table summarizes the information about the Docking Score and Confidence Score of the 1 to 4 molecular docking models of the cTnI protein and Tro4 and Tro6 aptamer, based on data retrieved from the HDOCK web server. It seems that Model 1 indicates the highest affinity and binding probability between both aptamers and cTnI protein.

Aptamer-based ELASA assay

To evaluate the binding capabilities of the aptamers to cTnI, direct ELASA assays were performed. Both Tro4 and Tro6 demonstrated significant binding to cTnI, confirming their suitability for detection applications. Optimization experiments identified 10µM biotinylated Tro4 and 5µM thiolated Tro6 as the optimal concentrations for the direct ELASA assay. The results validated the high affinity of the aptamers for cTnI,setting the stage for their integration into a sandwich-type assay for enhanced specificity and sensitivity (Figure 3). A sandwich ELASA platform was designed to improve detection performance by leveraging the combined binding capabilities of paired aptamers. Biotin was conjugated to the 5’ end of the capture aptamer (Tro4) to facilitate streptavidin binding without interfering with cTnI recognition. Thiol was conjugated to the 5’ end of the detection aptamer (Tro6) to enable HRP conjugation, ensuring effective signal generation.

Calibration and sensitivity

A calibration curve was constructed to evaluate the assay’s quantitative performance, correlating

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absorbance with cTnI concentrations ranging from 0.1 to 22 ng.mL. The resulting equation, y=0.0842ln(cTnI concentration)+0.3585y=0.0842\ln(cTnI.concentration)+0.3585y=0.0842ln(cTnI.concentration)+0.3585 (R^2=0.94), demonstrated strong linearity across the tested range (Figure 4). The calculated limit of detection (LOD) for the assay was 0.10 ng.mL, indicating its sensitivity in detecting low cTnI concentrations.

Clinical serum sample analysis

The aptamer-based sandwich ELASA assay was applied to the analysis of 52 clinical serum samples, including 27 samples from AMI patients and 25 from non-AMI individuals. Serum samples were also evaluated using a commercially available antibody-based ELISA kit (Monobind, USA).

Results from the aptamer-based assay showed a strong correlation with those obtained using the antibody-based ELISA kit, demonstrating the reliability and clinical applicability of the aptamer-based detection method. The findings clearly validated the potential of this innovative assay as a viable and efficient alternative to conventional antibody-based methods for detecting cTnI, which demonestrated in Table 1. (Table 1).

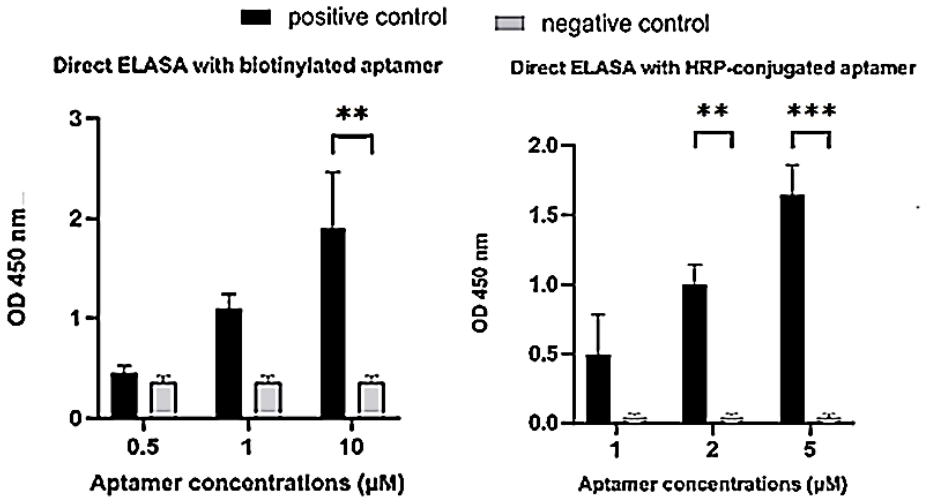

Figure 3. Screening of two aptamers binding ability against cTnI. Direct ELASA was used to screen two aptamer sequences for the detection of cTnI. Different concentrations of both aptamers were used to find the optimum concentration and it was shown that concentrations of 10 and 5µM were considered as the optimum.

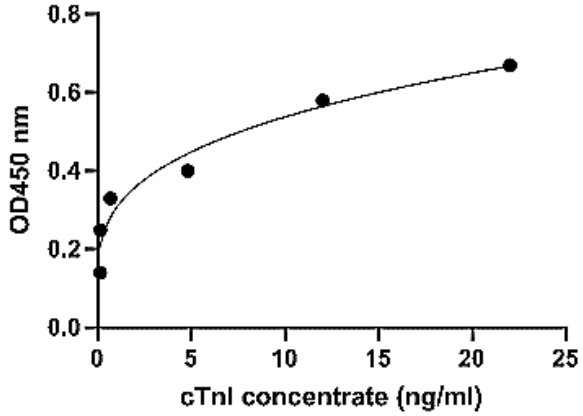

Figure 4. A calibration curve between absorbance and cTnI concentration from 0.1 to 22ng/Ml. To determine the linearity of the aptamer-based ELISA method, a sample with a specific concentration was diluted as 1.5, 1.10, 1.25, 1.175, 1.875, and 1.1000. The fitted linear equation is y=0/0842ln (cTnI concentration)+0/3585 (R^2=0.94).

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Table 1. Analysis of cTnI in AMI samples with the aptamer sandwich assay and comparison with monobind Elisa kit

Assay type	Results	N. of samples
Monobind ELISA kit	Total number of positive	27
	Total number of negative	25
Aptamer-based Sandwich ELASA assay	True positive	3
	False positive	4
	True negative	25
	False negative	-

In total, 52 samples were evaluated: 27 samples from patients with AMI and 25 samples from patients with no AMI. There were 31 males and 21 females with an average age of 54 and 42 years, respectively. The cTnI in all serum samples was measured with an Aptamer-based sandwich assay and compared the results with a commercially available antibody-based ELISA kit.

Discussion

Cardiac troponin I is a key biomarker for cardiac myocardial injury, particularly in the diagnosis of AMI. Its specificity and sensitivity for myocardial damage make it superior to many traditional markers. After AMI, cTnI levels peak within a few days and remain elevated for an extended period, facilitating timely and accurate diagnosis (17, 18). These attributes have established cTnI as a cornerstone for AMI diagnosis in clinical practice. The use of aptamers in diagnostic applications has gained attention due to their high specificity, stability, and ease of synthesis compared to traditional antibodies. However, for aptamers to achieve widespread clinical use, their integration into standard diagnostic platforms such as ELISA, electrochemiluminescence assays, and rapid diagnostic tests must be further optimized. This study focused on developing an aptamer-based ELASA (Enzyme-Linked Aptamer Sandwich Assay) for the detection of cTnI, demonstrating its potential as an alternative to antibody-based methods. Though our study has primarily focused on in silico validation of the aptamers' binding affinity to cardiac troponin I, future experimental validation is essential for confirming the docking predictions. Techniques such as SPR or EMSA will be employed to validate the binding kinetics and specificity of the aptamers, providing further support for their potential diagnostic applications. The docking scores provided an initial framework for selecting aptamers based on their predicted binding affinity. These scores are calculated based on the free energy of binding, which accounts for intermolecular forces such as hydrogen bonds and van der Waals interactions. While these predictions are valuable, they do not fully account for the complexities of biological systems, such as conformational flexibility, solvent effects, and potential off-target interactions. The observed correlation between docking scores and ELASA results validates the utility of docking simulations as a pre-screening tool, but experimental confirmation remains essential.

In the direct ELASA format, cTnI was coated onto microplate wells, and the binding ability of biotinylated and thiolated aptamers (Tro4 and Tro6) was evaluated at various concentrations (0.5, 1, and 10µM for biotinylated aptamers; 1, 2, and 5µM for thiolated aptamers). This approach, adapted from methodologies used for detecting SARS-CoV-2 nucleocapsid protein and Toxoplasma gondii ROP18 protein (19, 20), provided a robust framework for screening aptamer-binding efficiency. The sandwich ELASA format further enhanced the assay's specificity and sensitivity. By utilizing the strong biotin-streptavidin interaction, cTnI was immobilized via biotinylated aptamers onto streptavidin-coated wells, forming a capture probe. The detection probe, consisting of a thiolated aptamer conjugated with horseradish peroxidase (HRP), enabled dual recognition of cTnI.

The results demonstrated 100% sensitivity and specificity, with a limit of detection (LOD) surpassing that of traditional methods, such as the one reported by Husile Bai et al. (16). These findings underscore the assay's potential for high-accuracy diagnostics. Characterization of these aptamers through in silico studies showed that Tro4 and Tro6 can form distinct stem-loop structures, which enable them to target non-overlapping, spatially distinct epitopes on cTnI. This structural arrangement ensures that both aptamers can bind simultaneously and non-competitively to different regions of the cTnI protein. The structural versatility of these aptamers facilitates their effective use in a sandwich ELISA platform. Buffer composition played a crucial role in assay performance, as the structural integrity and functionality of aptamers depend on appropriate folding conditions. Tris buffer, selected based on its prevalence in aptamer-related studies (13, 14), provided optimal pH conditions for aptamer

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stability. Additionally, magnesium chloride ($MgCl_2$) and potassium chloride (KCl) were essential for stabilizing aptamer secondary and tertiary structures, ensuring efficient target binding. The function of aptamers can be influenced by several factors, including low ionic strength, extreme pH conditions, and high temperature. These factors can alter the binding affinity and stability of aptamers. Therefore, optimizing assay conditions is crucial to maintaining the efficiency and reproducibility of aptamer-based assays. In our study, the aptamers demonstrated specific and simultaneous binding even in serum and plasma, indicating that their structural and spatial arrangements are well-suited for binding in biologically relevant settings. However, further optimization of assay conditions, particularly pH and ionic strength, is recommended to ensure reliable performance in diverse biological matrices.One of the significant advancements in this study was the conjugation of aptamers directly to HRP. Traditional approaches rely heavily on biotin-streptavidin systems for linking aptamers to enzymes. Although effective, these systems can introduce interference, particularly in complex biological matrices, and may result in less stable configurations under variable assay conditions. Direct chemical conjugation of aptamers to HRP offers several advantages, including greater stability, resistance to environmental changes, and reduced interference. In designing aptamer modification for conjugation to HRP, it is crucial to account for the aptamer's structure and ensure that the thiol functional group is positioned away from the aptamer's binding site. This strategy minimizes the risk of interference with the aptamer's folding and preserves its binding efficiency to the target. Moreover, optimizing the molar ratio of the sulfo-SMCC linker, HRP enzyme, and aptamer during the conjugation process is essential.

This optimization ensures efficient conjugation, maintaining both the catalytic activity of HRP and the high binding efficacy of the aptamer. These steps help mitigate potential trade-offs in preserving aptamer functionality while enhancing sensitivity and specificity in complex biological matrices, such as serum or plasma. Previous diagnostic studies, such as those for LMBV by Zhang et al. and for Zika virus by Lee et al. (21, 22), utilized the biotin-streptavidin approach; however, this study demonstrates that direct enzyme conjugation is a viable and potentially superior alternative. To our knowledge, this is one of the first reports employing this strategy for cTnI detection, offering a novel direction for aptamer-based diagnostics.The aptamers used in this study against cardiac troponin I show promise for diagnostic applications. Besides ELASA, these aptamers can be incorporated into sensitive biosensors or point-of-care diagnostic platforms, enabling rapid detection of cardiac troponin I levels. Aptamers provide several advantages over traditional antibodies, including ease of synthesis, stability, and cost-effectiveness, making them ideal candidates for use in resource-limited settings. Furthermore, the aptamer-based ELASA assay offers a promising alternative to antibody-based ELISA.

Aptamers are less susceptible to batch-to-batch variability and exhibit superior chemical stability, allowing for more consistent and reliable diagnostic performance. This is particularly advantageous for point-of-care testing and applications in resource-limited settings where consistent cold-chain logistics for antibody preservation are challenging. In conclusion, this study successfully developed a sandwich ELASA assay for the detection of cTnI, demonstrating high sensitivity and specificity. The selection of aptamers Tro4 and Tro6 from Changill Ban et al.'s study provided an effective basis for assay development. Additionally, the innovative use of HRP-conjugated aptamers represents a significant advancement in aptamer-based diagnostics, addressing limitations associated with traditional biotin-streptavidin systems. The findings from this study highlight the potential of aptamer-based assays for clinical applications, particularly in the diagnosis of AMI. Future studies should explore the scalability of this approach, its performance in complex biological samples, and its integration into automated diagnostic platforms to facilitate clinical translation.

Acknowledgments

The present study was financially supported by a grant from the Deputy of Research, Shahid Beheshti University of Medical Sciences and the financial support of the research and development department of Arghavan Teb Kavian Company.

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[DOI: 10.22088/IJMCM.BUMS.14.2.705]

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[DOI: 10.22088/IJMCM.BUMS.14.2.705]