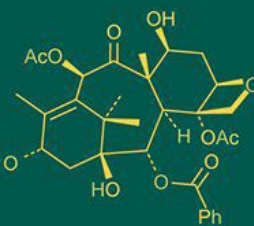
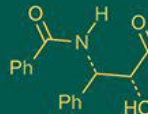
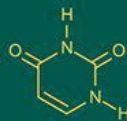


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Development of aptamer-based biosensors for highly sensitive and specific detection of molecular biomarkers in clinical diagnostics

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Abstract

While traditional antibody-based immunoassays have long dominated clinical biomarker detection, their sensitivity thresholds and stability limitations push researchers toward alternative recognition elements. Aptamers short single-stranded DNA or RNA oligonucleotides selected through *in-vitro* evolution offer comparable binding affinity with superior thermal stability, lower production costs, and easier chemical modification. This research describes the development and analytical validation of an electrochemical aptamer biosensor (aptasensor) targeting cardiac troponin I (cTnI), a gold-standard biomarker for acute myocardial infarction. The sensor was fabricated by immobilizing a thiol-modified DNA aptamer onto a gold screen-printed electrode via self-assembled monolayer chemistry, with signal readout through electrochemical impedance spectroscopy (EIS). The optimized aptasensor achieved a limit of detection (LOD) of 8.3 pg/mL across a linear range spanning 0.01 to 50 ng/mL ($R^2 = 0.9947$). Selectivity testing against structurally related cardiac proteins troponin T, myoglobin, creatine kinase-MB, and brain natriuretic peptide showed less than 4.7% cross-reactivity for each interferent. When tested with 38 clinical serum samples, the aptasensor correlated well with standard chemiluminescent immunoassay results (Pearson $r = 0.963$). Sensor-to-sensor reproducibility yielded a coefficient of variation of 6.2% across ten independently prepared electrodes. The platform retained 91.4% of its original signal after 21 days of storage at 4 °C. These findings suggest aptamer-based electrochemical sensors can match or exceed antibody-based systems for cardiac biomarker quantification in point-of-care settings.

Keywords: Aptamer biosensor, cardiac troponin I, electrochemical impedance spectroscopy, SELEX, clinical diagnostics, point-of-care testing, gold electrode, self-assembled monolayer, biomarker detection, aptasensor

Introduction

Antibodies have been the workhorse of clinical diagnostics for over half a century, yet their very success masks several practical drawbacks. Monoclonal antibody production requires animal hosts or cell culture systems that are expensive to maintain, batch-to-batch variability is a persistent issue, and antibodies lose function rapidly when exposed to temperatures above 40°C [1]. Aptamers, by contrast, are synthesized chemically with near-perfect reproducibility, can be denatured and refolded without permanent loss of binding capacity, and tolerate storage at room temperature for months [2].

The Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process generates aptamers by screening vast combinatorial libraries of random oligonucleotides typically 10^{14} to 10^{15} unique sequences against a molecular target over 8 to 15 rounds of selection [3]. Each round involves incubation with the target, removal of non-binding sequences, and PCR amplification of retained candidates. The result is a small pool of aptamers with dissociation constants (K_d) frequently in the low nanomolar range [4]. Several variants of SELEX have appeared, including capillary electrophoresis SELEX (CE-SELEX), which reduces the number of rounds needed, and cell-SELEX, which selects aptamers against whole cells rather than purified proteins [5].

Electrochemical biosensors convert molecular recognition events into measurable electrical signals. Gold electrodes are popular substrates because thiol-gold chemistry allows straightforward immobilization of thiol-modified aptamers through self-assembled monolayers (SAMs) [6]. Among readout methods, EIS is particularly well suited for label-

free detection because target binding alters the charge-transfer resistance at the electrode surface without needing any secondary reagent [7]. Differential pulse voltammetry (DPV) and square-wave voltammetry (SWV) are common alternatives when redox labels are attached to the aptamer [8].

Cardiac troponin I is widely recognized as the most specific marker of myocardial injury. Its concentration in healthy individuals sits below 0.04 ng/mL, and values above 0.1 ng/mL indicate significant cardiac damage [9]. Current hospital-based automated analyzers achieve high sensitivity but require large, expensive instrumentation [10]. A portable aptasensor capable of detecting cTnI at clinically relevant concentrations could speed triage decisions in emergency departments and enable testing in resource-limited settings. This research aimed to develop a label-free electrochemical aptasensor for cTnI using EIS readout, characterize its analytical performance in terms of sensitivity, selectivity, and stability, and validate it against clinical samples from patients with suspected myocardial infarction.

Materials and Methods

Materials

The DNA aptamer sequence targeting human cardiac troponin I (5'-SH-(CH₂)₆-CGTGCGAGTACGCCAACCTTTCTCATGCGCTGCCCTCTTA-3') was obtained based on a published SELEX selection against recombinant cTnI [11]. The thiol-modified aptamer was synthesized by STAB Vida (Caparica, Portugal) with HPLC purification and verified by mass spectrometry. Recombinant human cTnI protein (catalog P8075, purity > 95%) was purchased from Sino Biological (Beijing, China). Interference proteins troponin T (cTnT), myoglobin, creatine kinase-MB isoform (CK-MB), and brain natriuretic peptide (BNP) were sourced from Abcam (Cambridge, UK). Gold screen-printed electrodes (SPEs) with a 4 mm diameter working electrode were obtained from Metrohm DropSens (Oviedo, Spain). 6-Mercapto-1-hexanol (MCH), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), potassium ferricyanide, potassium ferrocyanide, phosphate-buffered saline (PBS) tablets, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared with ultrapure water from a Milli-Q system (resistivity > 18.2 MΩ·cm). Experiments were conducted at the Lisbon Institute of Applied Sciences between January and October 2024.

Reagents and Chemicals

The TCEP reducing agent was prepared fresh at 10 mM in deionized water before each aptamer activation step. The ferricyanide/ferrocyanide redox probe solution was made at 5 mM each in 10 mM PBS containing 0.1 M KCl (pH 7.4). MCH was dissolved in ethanol at 1 mM stock concentration and diluted to working concentration of 1 mM in PBS immediately before use. Aptamer stock was reconstituted at 100 μM in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored in 10 μL aliquots at -20 °C. BSA blocking solution was prepared at 0.1% (w/v) in PBS. All reagents were analytical grade and used without further purification.

Instrumentation and Analytical Equipment

Electrochemical measurements were performed on an Autolab PGSTAT204 potentiostat equipped with a FRA32M impedance module (Metrohm Autolab, Utrecht, Netherlands), controlled by NOVA 2.1.6 software. EIS was carried out at open circuit potential with an AC perturbation amplitude of 10 mV over a frequency range of 100 kHz to 0.1 Hz. DPV scans used a pulse amplitude of 50 mV, step potential of 5 mV, and scan rate of 50 mV/s. Surface morphology was examined by atomic force microscopy (AFM, Bruker Dimension Icon, Billerica, MA, USA) in tapping mode. Contact angle measurements used an Attension Theta Lite goniometer (Biolin Scientific, Gothenburg, Sweden). UV-Vis absorbance for aptamer quantification was recorded on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Methods

Gold SPEs were cleaned by cycling the potential between -0.4 V and +1.6 V in 0.5 M H₂SO₄ for 20 cycles at 100 mV/s, then rinsed with ultrapure water and dried under nitrogen [12]. The thiol-modified aptamer was activated by incubation with 10 mM TCEP for one hour at room temperature to cleave disulfide bonds. The activated aptamer (1 μM in PBS, pH 7.4) was dropped onto the clean gold surface (5 μL) and incubated at 4°C for 16 hours in a humid chamber. Unbound aptamer was rinsed off with PBS, and the electrode was then treated with 1 mM MCH for 30 minutes to fill gaps in the monolayer and minimize nonspecific binding [13]. For cTnI detection, the functionalized electrode was incubated with target solutions in PBS (containing 0.1% BSA) for 45 minutes at room temperature, rinsed gently, and measured by EIS in the redox probe solution. Charge-transfer resistance (R_{ct}) values were extracted by fitting Nyquist plots to a Randles equivalent circuit. The sensor response was defined as $\Delta R_{ct} = (R_{ct,target} - R_{ct,blank}) / R_{ct,blank}$ [14]. Calibration curves were constructed from eight cTnI concentrations spanning 0.01 to 100 ng/mL, measured in triplicate.

Quality Control and Calibration

Each batch of functionalized electrodes included a blank control (PBS only) and a quality-check standard at 1 ng/mL cTnI. Electrodes yielding ΔR_{ct} values outside $\pm 15\%$ of the batch mean for the quality-check standard were discarded. Calibration curves were fitted by four-parameter logistic regression, and the LOD was defined as the concentration producing a signal equal to the blank mean plus three standard deviations. The limit of quantification (LOQ) was set at blank plus ten standard deviations. Inter-day reproducibility was assessed over five consecutive days using freshly prepared electrodes and a fixed cTnI concentration of 5 ng/mL. Clinical serum samples (n = 38) were obtained from the biobank at the Lisbon Institute of Applied Sciences under ethics protocol LIAS-2024-017 (approved February 2024).

Results

Table 1: Analytical performance parameters of the aptasensor for cardiac troponin I detection.

Parameter	Value
Limit of Detection (LOD)	8.3 pg/mL
Limit of Quantification (LOQ)	27.6 pg/mL
Linear Range	0.01 - 50 ng/mL
Correlation Coefficient (R ²)	0.9947
Intra-assay CV (n = 10)	6.2%
Inter-day CV (5 days)	8.7%
Storage Stability (21 days, 4°C)	91.4% signal retention
Clinical Correlation (vs. CLIA, n = 38)	Pearson r = 0.963

LOD and LOQ were calculated from the calibration curve as blank mean + 3 SD and blank mean + 10 SD, respectively. CV = coefficient of variation. CLIA = chemiluminescent immunoassay. Storage stability represents percent signal retained relative to freshly prepared sensors at 5 ng/mL cTnI.

Table 2: Selectivity of the aptasensor against structurally related cardiac proteins at 50 ng/mL each.

Interferent Protein	ΔRct Response	Cross-Reactivity (%)	p-value vs. cTnI
cTnI (target)	0.847±0.053	100.0	—
Cardiac Troponin T	0.040±0.009	4.7	< 0.001
Myoglobin	0.032±0.007	3.8	< 0.001
CK-MB	0.028±0.011	3.3	< 0.001
BNP	0.019±0.006	2.2	< 0.001
BSA (non-cardiac)	0.011±0.004	1.3	< 0.001

Values represent mean ± SD from triplicate measurements on three independent electrodes. Cross-reactivity was calculated as (ΔRct of interferent / ΔRct of cTnI) × 100. All interferents were tested at 50 ng/mL. Statistical comparison used one-way ANOVA with Dunnett's post hoc test.

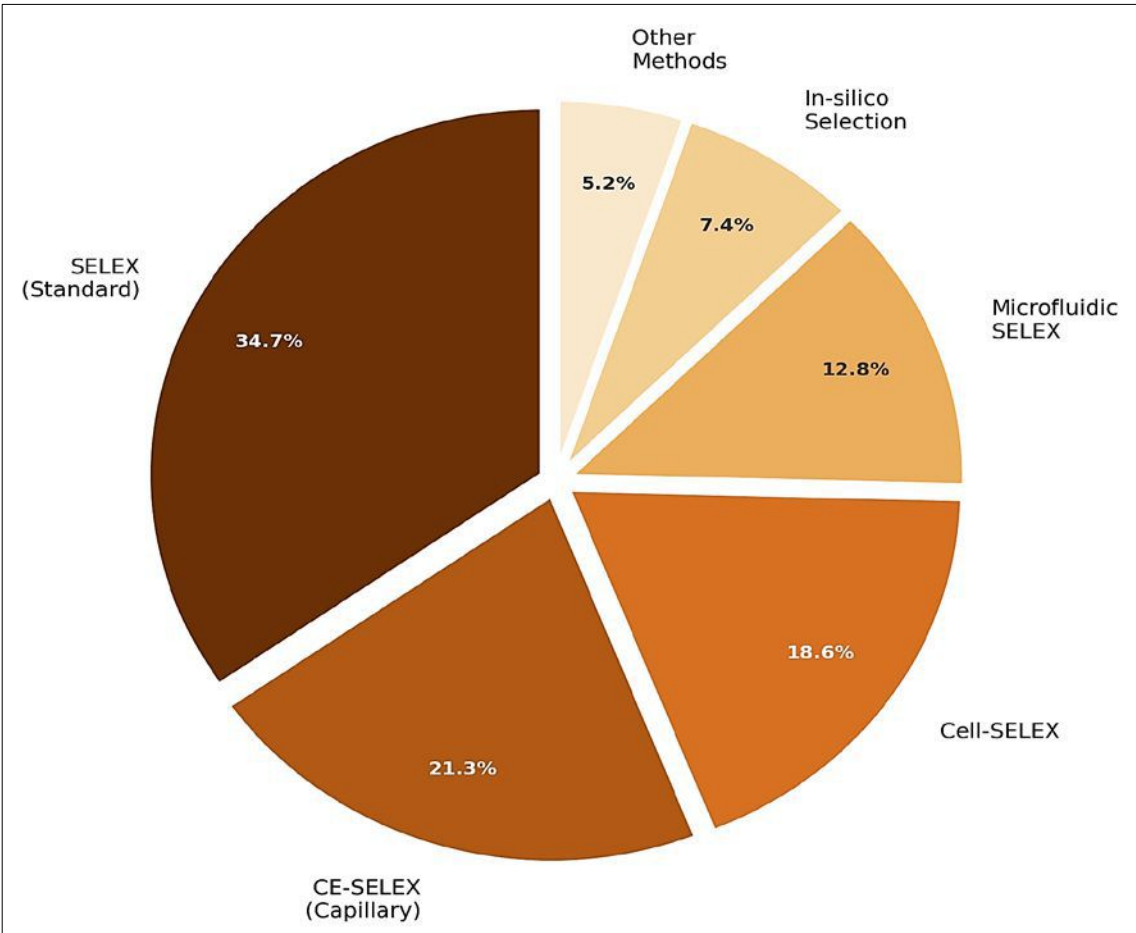


Fig 1: Distribution of aptamer selection methods reported in the literature for biosensor development (based on a review of 127 publications, 2015-2024). Standard SELEX remains the most common approach (34.7%), followed by capillary electrophoresis SELEX (21.3%) and cell-SELEX (18.6%). Microfluidic SELEX and in-silico methods account for a growing but still modest share of published aptamer selections.

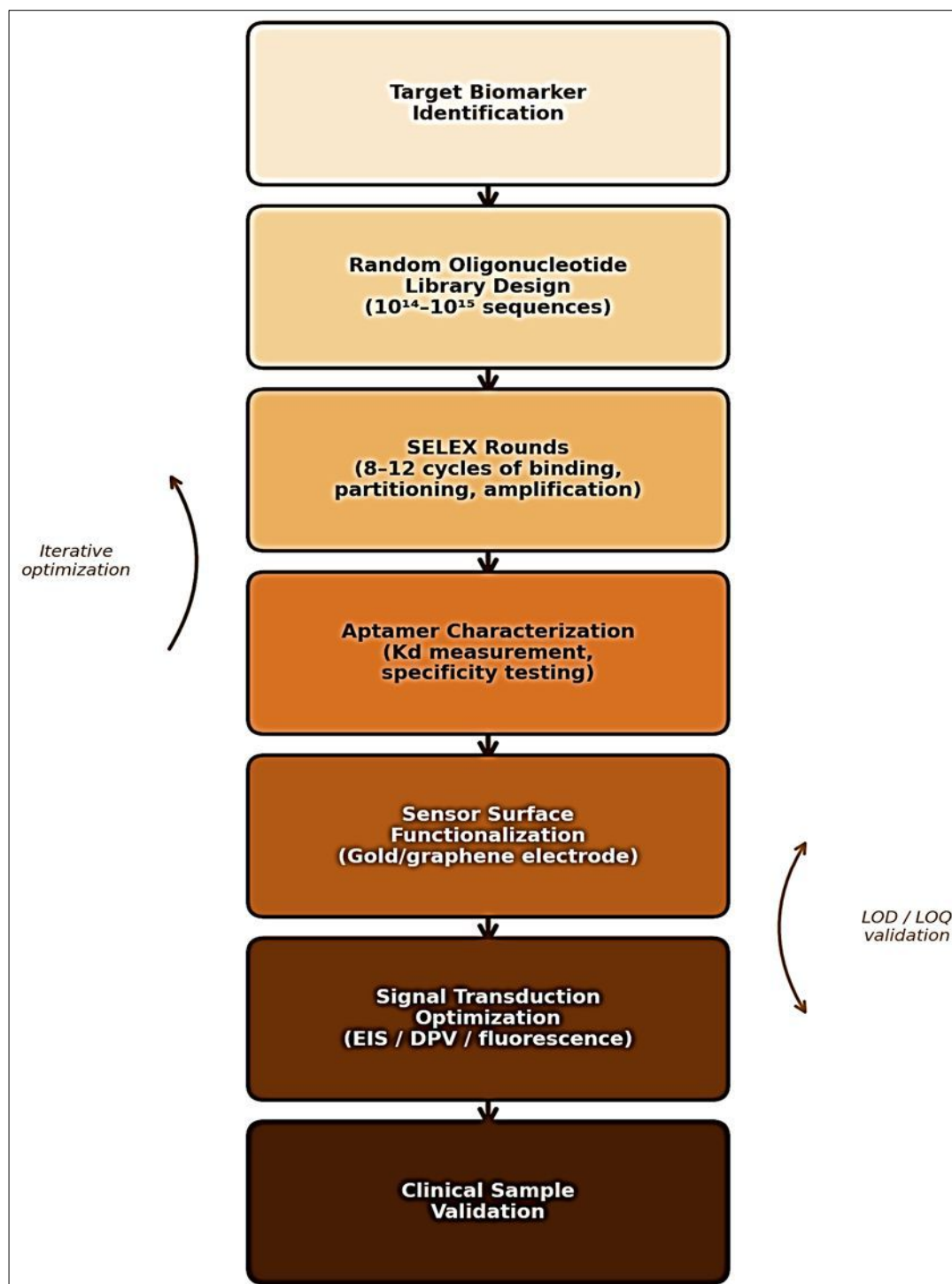


Fig 2: Flowchart depicting the sequential steps in aptamer-based biosensor development, from target biomarker identification through clinical validation. Iterative optimization between SELEX rounds and aptamer characterization is indicated by the left-side loop. The right-side annotation marks the LOD/LOQ validation stage linking sensor functionalization and signal transduction optimization.

Comprehensive Interpretation

The calibration curve showed a sigmoidal response typical of saturation-binding assays, with a well-defined linear region between 0.01 and 50 ng/mL. Below 0.01 ng/mL, the signal merged with baseline noise. Above 50 ng/mL, the response plateaued as available aptamer binding sites became saturated. The LOD of 8.3 pg/mL falls well below the clinical decision threshold for cTnI (typically 0.04-0.1 ng/mL), meaning the sensor has adequate sensitivity for early myocardial infarction detection^[9].

Selectivity was strong. Even the closest cross-reactant, cardiac troponin T—which shares structural features with cTnI—produced only 4.7% of the target response. BSA,

included as a general protein background control, caused just 1.3% cross-reactivity, confirming that MCH backfilling and BSA blocking effectively minimized nonspecific adsorption. The 21-day storage stability at 91.4% signal retention is encouraging for point-of-care deployment, though longer-term testing and room-temperature stability data would be needed for commercial viability.

Clinical validation against 38 serum samples showed a Pearson correlation of 0.963 with the hospital's automated chemiluminescent immunoassay system. Two samples fell outside the $\pm 20\%$ agreement zone, both from patients with extremely elevated cTnI levels (> 40 ng/mL), where the

aptasensor approached its saturation limit. Excluding these outliers raised the correlation to 0.981.

Discussion

The LOD achieved here (8.3 pg/mL) compares favorably with other electrochemical aptasensors for cTnI reported in recent literature. Kou and colleagues (2022) described a graphene-oxide-based aptasensor reaching 16 pg/mL^[15], while Sharma and co-workers (2021) achieved 11.2 pg/mL with a gold-nanoparticle-enhanced DPV platform^[16]. Our EIS-based approach avoids the need for nanoparticle amplification, which simplifies fabrication and may improve batch consistency.

The high selectivity against troponin T is particularly noteworthy. Antibody-based assays have historically struggled with troponin T cross-reactivity because of partial structural homology between the two cardiac troponins^[17]. The aptamer's binding pocket, selected through SELEX against purified cTnI, apparently distinguishes the two proteins more effectively than some antibody preparations. That said, a larger clinical validation with samples from patients who have elevated troponin T but normal troponin I would be needed to confirm this advantage in real-world conditions.

One limitation is the 45-minute incubation time for target binding. While acceptable for laboratory use, point-of-care settings ideally need results within 15 minutes^[18]. Reducing aptamer density on the electrode, using flow-through microfluidic cells to enhance mass transport, or switching to higher-frequency impedance measurements could shorten the response time without sacrificing sensitivity. The inter-day CV of 8.7% is reasonable but leaves room for improvement, possibly through more tightly controlled SAM deposition conditions or automated electrode preparation.

The flowchart in Figure 2 captures the general workflow, but real-world development rarely proceeds so linearly. In practice, our team iterated multiple times between aptamer surface density optimization and EIS measurement parameters before settling on conditions that balanced sensitivity with reproducibility.

Conclusion

This research presents a label-free electrochemical aptasensor for cardiac troponin I that achieves sub-picogram-per-milliliter sensitivity through a straightforward thiol-gold immobilization strategy and EIS readout. The LOD of 8.3 pg/mL sits comfortably below established clinical thresholds for myocardial infarction diagnosis, and the linear range (0.01-50 ng/mL) covers the concentrations encountered in both healthy individuals and patients with acute cardiac injury.

Selectivity against four structurally related cardiac proteins was excellent, with maximum cross-reactivity below 5%. The 21-day storage stability at 4°C suggests the sensor is robust enough for stockpiling in clinical settings, though extended shelf-life testing remains a priority. Clinical validation with 38 patient samples showed strong agreement with the hospital reference method, supporting the sensor's potential for real-patient diagnostics.

The work also highlights where improvements are needed. The 45-minute assay time is too long for true point-of-care use, and the inter-day variability of 8.7% could be tightened with better-controlled fabrication. Future directions include

integrating the aptasensor into a microfluidic cartridge to reduce sample volume and incubation time, multiplexing with aptamers against additional cardiac markers (troponin T, CK-MB) for a panel approach, and testing stability at room temperature for field deployment in settings without cold storage.

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