

Article

Chemiluminescence Biosensor for the Determination of Cardiac Troponin I (cTnI)

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Abstract: Cardiac vascular diseases, especially acute myocardial infarction (AMI), are one of the leading causes of death worldwide. Therefore cardio-specific biomarkers such as cardiac Troponin I (cTnI) play an essential role in diagnostics. In order to enable rapid and accurate measurement of cTnI with the potential of online measurements, a proof of concept of a chemiluminescence-based biosensor is presented. A flow cell was designed and combined with a sensitive CMOS camera allowing an optical readout. In addition, a microfluidic setup was established, and cTnI was determined selectively. The biomarker cTnI was expressed in *E. coli*, and its characterization and correct folding were investigated by different analytical methods. This recombinant cTnI was used for enzyme-linked immunosorbent assays (ELISA), calibrated against commercially available recombinant cTnI, and applied for the biosensor measurements. Based on chemiluminescence detection, the biosensor was successfully tested, and the cTnI biomarker could be reproducibly determined in buffer, spiked blood serum, and plasma.

Keywords: acute myocardial infarction (AMI); cardiac Troponin I (cTnI); chemiluminescence; biosensor; luminol; monoclonal antibody; flow injection assay; microfluidic system; monolithic column; protein expression

1. Introduction

In our modern society with its aging population, heart diseases remain a major cause of morbidity and mortality, with 1.19 million deaths per year in 2017 and € 169 billion of estimated related costs for the health care systems [1]. Guidelines on the prevention, diagnosis, and treatment of heart diseases such as acute coronary syndrome have been published by the European Society of Cardiology (ESC) [2,3]. In the case of acute myocardial infarction (MI), cardiac troponin (cTn) is the most important biomarker for the diagnosis [3,4]. cTn is a protein in the heart muscle released into the bloodstream when muscle cells are damaged. The release of cTn into the blood is not exclusive to MI but can occur in various acute and chronic conditions. However, specific to MI is the rise and fall of cTn levels over time. Therefore, a diagnosis is based on the cTnI concentration at baseline level and changes in its concentration usually over 2-6 hours, depending on the sensitivity of the assay used, as well as considering other risk factors to distinguish between chronic conditions and MI [5]. A point-of-care test (POCT), which can already be used in the ambulance, delivering quasi-continuous results for the cTnI concentration, would enable the fast evaluation of cTnI concentration changes in the blood. Thus, it allows a quicker rule-in/rule-out decision during the presentation at the hospital. For clinical laboratories and POCTs, all relevant forms of cTnI must be recognized. Therefore,

epitopes should be selected to be as unaffected as possible by posttranslational modifications (PTMs) such as phosphorylation [6] or glycation [7], or truncation [8]. Also, cTnI should be detected in complexes like cTn IC or cTn ITC [6].

In order to achieve the fast and reliable detection of cTnI, a biosensor was developed within the project 18HLT10 CardioMet [9], a project funded in the framework of the European Metrology Program for Innovation and Research (EMPIR). A biosensor previously demonstrated for detecting cocaine [10], and TNT [11] showed high sensitivity. In this work, instead of fluorescence detection, chemiluminescence was used to reduce the background further and improve sensitivity; the principle is shown in Figure 1. cTnI-specific antibodies labeled with horseradish peroxidase (HRP) were mixed with the sample potentially containing cTnI. The mixture then passes an affinity column consisting of a borosilicate glass monolith functionalized with a surrogate peptide of the cTnI epitope targeted by the antibody. If the sample contains cTnI, the antibody labeled with the enzyme is blocked and cannot bind to the peptide on the column (competition) and passes through the detector. This results in a signal by a chemiluminescence substrate reaction, and light is emitted. In contrast, if no cTnI is present in the sample, the labeled antibody will quantitatively bind to the surrogate peptide on the column, no enzyme-labeled antibody will exit the column, and no signal can be detected. In this work, an antibody and peptide combination has been tested as a proof of concept to achieve the best sensitivity and selectivity for cTnI.

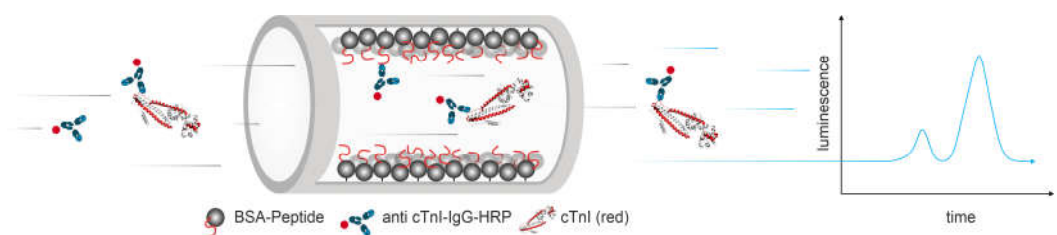


Figure 1. Biosensor principle: Anti-cTnI-IgG-HRP binds to the antigen cTnI (4y99) if present in the sample and can no longer interact with the epitope-functionalized column. Depending on the antigen concentration, the antigen-antibody complex elutes, and the chemiluminescence intensity can be detected by substrate mixing.

When techniques such as conventional POCTs, biosensors, and laboratory-based measurements are used, the results may deviate significantly. Therefore, for a reliable diagnosis, the comparability of the results is of the utmost importance. Traceability to the International System of Units (SI) is the preferable way to ensure comparability of the results. For this purpose, a commutable reference or calibration material with assigned values traceable to the SI [12,13] can be used.

However, for most clinical markers, such calibration materials do not exist. Previous studies have provided valuable information on recombinant expression and purification of various constructs of cTn [14,15]. Using these proteins as calibration materials can pose challenges related to stability and purity. In our case, recombinant human cTnI and other troponins were prepared, characterized, compared with a commercial material, and used as a calibrant.

2. Materials and Methods

2.1. Reagents, Buffers, Materials, and Equipment

The peptide Pep188-199 (NH₂-CGGGDWRKNIDALSG-NH₂, 1548.1 g/mol, 95.7 % purity) was synthesized by peptide & elephants (Hennigsdorf, Germany), bovine serum albumin (BSA) >98% (A7906), BSA protease-free >98% (A7030), ProClin300 (8912-U), 4-[4-(Dimethylamino)phenylazo]benzoic acid N-succinimidyl ester (SBAP, 09278-25MG-F), (3-Aminopropyl)triethoxysilane (440140), sinapic acid (85429-1G) and Mucosol (Z637203) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Phosphate-buffered saline (PBS)- (1X Dulbecco's)-Powder and tris base (77-86-1) were received from AppliChem GmbH (Darmstadt, Germany). Transparent and white, flat-bottomed high binding 96-well microtiter plates were ordered from Greiner Bio-One (Frickenhausen, Germany), Zeba™ Micro Spin desalting columns (7 kDa MWCO, 75 µL), high sensitivity Neutravidin-HRP (31030) and EZ-Link™ NHS-PEG₁₂-Biotin (21312), trifluoroacetic acid 99.5% (TFA, 85183) and sodium cyanoborohydride (168552500) were obtained from Thermo Scientific (Waltham, USA), PD-10 desalting columns packed with Sephadex G-25 resin (15 mL, 5 kDa MWCO) was acquired from Cytiva (Washington, DC, USA), monoclonal anti-human troponin I antibody (Anti-h cTnI 9707 SPTN-5) was received from Medix Biochemica (Espoo, Finland). HRP Conjugation Kit - Lightning-Link (ab102890) was contributed from Abcam (Amsterdam, Netherlands). The chemiluminescent substrate (SuperSignal West Pico PLUS, 34580) was bought from Thermo Scientific (Waltham, USA), Tween 20 (37470.01) from Serva (Heidelberg, Germany), absolute ethanol (2246), acetonitrile 99.95% (ACN, 2697) and 150 mL filtration cups with 0.2 µm PES membrane from Th. Geyer (Renningen, Germany). PlateBlock (112500) and LowCrossBuffer (100500) were received from Candor Bioscience (Wangen, Germany). Peroxidase-conjugated AffiniPure goat anti-mouse IgG (115-035-003) and AffiniPure goat anti-mouse IgG (115-005-164) were purchased from Jackson ImmunoResearch Europe Ltd (Cambridge House, UK). Polyclonal goat anti-human Cardiac Troponin I (4T21/2), recombinant (rec.) human cTnI (8RTI7) and Troponin I free serum (8TFS) was provided by HyTest Ltd (Turku, Finland). Lyophilized plasma (1 g per 10 mL of ultra-pure water for reconstitution) was purchased from the German Red Cross (Berlin, Germany). 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (SeramunBlau fast 2, S100-TMB) was ordered from Seramun Diagnostica GmbH (Heidesee, Germany), and sulfuric acid (15624970) from Fisher Scientific GmbH (Schwerte, Germany). Vitrapor5 glass monoliths were purchased from Robu (Hattert, Germany). Glutaraldehyde 50% in water (111-30-8) was ordered from Merck KGaA (Darmstadt, Germany).

The microfluidic flow cell was designed and produced in-house; details are shown in the supplementary materials (drawing.pdf). Polyoxymethylene (POM) was purchased from Grünberg Kunststoffe GmbH (Berlin, Germany) and used for the subtractive manufacturing of the cell. Planoconvex and planoconcave lenses (49875 and 48334; 9 mm diameter; 12 mm focal length) were purchased from Edmund Optics Inc. Immersion oil (Immersol 518 N) was received from Zeiss (Oberkochen, Germany). For the incubation loop, a PTFE tubing with an inner diameter of 1 mm was purchased from Th. Geyer (Renningen, Germany). The camera detector QHY174M-GPS was ordered from QHYCCD (Beijing, China). The microfluidic micromixer (10000759) was acquired from Microfluidic ChipShop (Jena, Germany), the injection valve (5067-4158) from Agilent (Santa Clara, CA, USA), and a Fusion 4000X syringe pump was ordered from Chemyx (Stafford, TX, USA). Omnifix 30 mL (6303643) and 50 mL (4665914) Luer slip syringes were purchased from Braun (Melsungen, Germany), and 2 mL Luer slip syringes (7644125) from Th. Geyer (Renningen, Germany).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry was performed on a Bruker Autoflex Max MS. Chemiluminescence was measured with a Synergy H1 spectrometer, and the absorbance was recorded with an Epoch2 Photometer from Biotek (Winooski, VT, USA). Data evaluation was performed with SharpCap Astro Capture Software Version 3.2.6482.0 (AstroSharp Limited, UK), and

Python 3.7 in Anaconda (Austin, TX, USA) and Origin 2018G (Northampton, MA, USA). Ultrapure water (MilliQ) was supplied by a Milli-Q Synthesis A10 system (Merck, Germany).

Most general chemicals, including ultra-pure imidazole, β -mercaptoethanol (β ME), NaCl, Tris, CaCl_2 , Na_2HPO_4 , KH_2PO_4 , IPTG, Arginine, Glutamic acid, and antibiotics for prokaryotic cells, chloramphenicol were purchased from Carl Roth. Ampicillin was supplied by AppliChem. Tris(2-carboxyethyl)phosphine (TCEP) was ordered from AbMole Bioscience. Tris-buffer (2 M), CaCl_2 (1 M), and NaCl (5 M) stocks were prepared in-house using the chemicals bought from Carl Roth. Alkaline phosphatase, protein kinase A, competent cells NEB5 α or BL21 (DE3), T7 express or T7 express lysY/Iq were bought from New England Bio-labs. Plasmid DNA purification kits were ordered from PEQLAB Biotechnologies or Qiagen. TEV protease and PKA (protein kinase A) were expressed and purified from an in-house construct [16]. HisTrap excel, HisTrap FF, HiTrap Heparin HP columns, and HiLoad 26/600 Superdex S200 pg or S75 pg are from GE Bioscience/Cytiva. Pre-casted NuPAGE 4-12% Bis-Tris gels were purchased from Novex life technologies. PageRuler™ Unstained Protein Ladder (26614X4) was ordered from Thermo Scientific (Waltham, USA). For isotopic labeling of proteins in prokaryotes, ^{15}N NH_4Cl was purchased from Sigma-Aldrich.

2.2. Peptide-Design and Peptide-BSA Conjugation

The cTnI epitope of the monoclonal mouse antibody clone 9707 was obtained from the corresponding data sheet of the supplier Medix Biochemica [17] and as published previously [18]. Based on this information, the following peptide amino acid sequence (Pep188-199) with a molecular weight of 1548.1 g/mol was chosen and synthesized by peptide & elephants GmbH: $\text{NH}_2\text{-CGGGDWRKNIDALSG-NH}_2$. The highlighted sequence indicates the C-terminal cTnI epitope (amino acid residues 190-195) of the antibody 9707. The peptide sequence includes an N-terminal cysteine as a conjugation site, a short glycine spacer, and an amidated C-terminus.

For the conjugation, 25 mg of BSA protease-free was dissolved in 2250 μL of 0.15 M PBS at pH 8. 4.625 mg of succinimidyl 3-(bromoacetamido)propionate (SBAP) was dissolved in 250 μL of DMSO and added to the BSA with a 40-fold molar excess relative to BSA. The reaction was performed in an overhead shaker at room temperature (light protected). After three hours, a PD MiniTrap G25 Desalting Column (with 5 kDa cut-off) was equilibrated with PBS, pH 8, and used to purify the activated protein by size exclusion chromatography. 2500 μL of the eluate was pipetted into 4.5 mg Pep188-199 (10-fold molar excess relative to eluted BSA). After overnight incubation at 4 °C, the conjugate was purified with a PD MiniTrap G25 Desalting Column (with 5 kDa cut-off), eluted with PBS pH 7.4, and stored at 4 °C. For long-term storage, 0.05 % of ProClin300 was added.

The successful conjugation was confirmed by MALDI-TOF mass spectrometry. 10 μL of the conjugate was desalted using a MicroSpin desalting column (7 kDa MWCO, 75 μL), equilibrated with purified water, and 1 μL of the eluate was spotted on the MALDI target, and 1 μL of 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid, SA, 10 mg/mL in purified water: ACN, 70:30 with 0.1% TFA) was added.

2.3. Column Preparation and Functionalization with Peptide-BSA Conjugate

The column was prepared as previously described [19]. The glass monolith was glued into a titanium shell and inserted into a column holder, as shown in Figure 2.



Figure 2. Engineering drawing of the column holder made by additive manufacturing with custom 1/16" PEEK fittings and monolithic affinity column in the center.

After column insertion, some cleaning steps, silanization with (3-aminopropyl)triethoxysilane, as described in Table S1, and functionalization with the peptide-BSA conjugate were performed. First, activation was conducted with 10 mL of 5 % glutaraldehyde in PBS (pH 7.4) pumped through the column at 0.2 mL/min and sealed afterward. Then, the incubation was performed overnight at room temperature. After washing, the column was functionalized with 3 mL at 0.01 mL/min of Peptide-BSA conjugate (around 2 mg/mL) and incubated overnight. Subsequently, 4 mL of NaCNBH₃ (50 mM in PBS pH 7.4) was pumped through the column at 0.1 mL/min. Finally, the surface was blocked by flushing the system with 4 mL of 1 % BSA (in 0.1 M Tris buffer and 50 mM NaCNBH₃ at pH 7.4) at 0.1 mL/min, followed by purging the column with PBS buffer, including 0.1% Tween20 and 0.1 % BSA, and finally with 80 % of ethanol. The column was stored with 80 % ethanol at 4 °C until use.

2.4. Troponin Expression in *E. coli* and cTnI Calibration

2.4.1 Constructs and molecular cloning

Individual coding sequences of each component of human cardiac troponin protein cTnI (1M...S210; NCBI Reference Sequence: NP_000354.4), cTnT (1M...K298), and cTnC (1M...E161) were synthesized at Genscript, either with additional NdeI and BamHI restriction sites or BsaI and XbaI restriction sites, at the N- and C-terminus respectively. Further, all gene sequences were optimized for their expression in *E. coli*. The synthesized genes were individually subcloned into a modified pET TEV vector (a modified pET-16b vector; Novagen [16]) and a pE-SUMO vector. Subcloning troponins into the pET TEV vector uses NdeI and BamHI restriction enzymes. It provided an N-terminal His tag, followed by a TEV protease cleavage sequence (Tobacco Etch Virus, TEV) and a short linker sequence in front of the desired gene. And subcloning troponins into the pE-SUMO vector is done by using BsaI and XbaI restriction enzymes, which resulted in an N-terminal His tag, followed by SUMO tag, in front of the desired gene. It enabled the achievement of native proteins without any additional linker amino acids when cleaved with SUMO protease.

To prepare the cTn complex, cTnC and cTnT were subcloned into a dual expression vector pACYC-Duet. The cTnC gene constructs mentioned before with restriction sites NdeI and BamHI at the N- and C-terminus is modified at the N-terminus to NcoI, retaining the BamHI at the C-terminus and cTnT gene construct with restriction sites NdeI and BamHI at the N- and C-terminus is modified at the C-terminus to KpnI, retaining the NdeI at the N-terminus. With the modified restriction sites, cTnC and cTnT were

subcloned in the pACYC-Duet vector at NcoI, BamHI, NdeI, and KpnI sites, respectively (Genscript).

2.4.2. Protein expression in E.coli and purification

For protein expression, the recombinant plasmids were transformed into either T7 express, BL21 (DE3) or Shuffle T7 express, or T7 express lysY/Iq according to the manufacturer's protocol (NEB). Transformed cells were plated and grown overnight on LB agar plates with either ampicillin (100 µg/mL) or ampicillin (80 µg/mL) and chloramphenicol (30 µg/mL). Single clones were picked and inoculated to perform test expressions and prepare glycerol stocks.

2.4.3. Test expression of the cTn constructs

The recombinant plasmids were transformed either into T7 express, BL21 (DE3), or Shuffle T7 express or T7 express lysY/Iq. With the transformants, test expressions were performed in a 10 mL LB medium with respective antibiotics. Cells were grown in a 37 °C incubator with 160 rpm, and when the cells reached an optical density of 0.6 - 0.8, they were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After three hours of expression at 37 °C, cells were harvested and analyzed by SDS-PAGE. cTn recombinant constructs transformed into T7 express lysY/Iq cells have more expression of the target gene. Therefore the cTn recombinants transformed into T7 express lysY/Iq were used for large-scale expressions.

2.4.4. Large-scale expression

Transformed cells were inoculated into 10 mL cultures (pre-pre-inoculum) of Luria broth or ¹⁵N-M9 medium, with respective antibiotics, and grown for 4 h at 37 °C. From this pre-pre-inoculum, 100 mL of overnight pre-cultures in Luria broth (LB, unlabeled) or ¹⁵N M9 medium (for ¹⁵N isotopic labeling) with respective antibiotics were prepared. Cells were either directly inoculated into two liters of LB medium with antibiotics or into two liters ¹⁵N M9 medium with antibiotics. Cultures were grown at 37 °C, and when the optical density reached 0.6 - 0.8, cells were induced with 1 mM IPTG, and expression was at 37 °C for four to five hours. After the expression, the cells were harvested by centrifugation (4000 rpm at 4 °C for 10 min), and the cell pellets were stored at -80 °C until use.

2.4.5. Protein purification and characterization

For protein purification, frozen cell pellets are thawed on ice and resuspended in their respective Ni-NTA binding buffer i.e. lysis or loading buffer for cTnC 50 mM Tris pH 8.2, 200 mM NaCl, 5 mM CaCl₂, 10 mM βME, 5 mM imidazole was used. For cTnI and cTnT 100 mM Tris-HCl, pH 8.2 buffer containing 1 M NaCl, 15 mM CaCl₂, 25 mM arginine, 25 mM glutamic acid, 10 mM βME, and 5 mM imidazole have been used. Mechanical cell disruption was performed with a microfluidizer processor, bypassing the cell suspension through the interaction chamber for 3-5 cycles at 15000 psi. Then the lysed cells were centrifuged at 16,000 rpm for 45 min at 4 °C. The cleared supernatant separated from the insoluble pellet was loaded onto either a His-Trap HP column (Cytiva) or HisTrap Excel (Cytiva) equilibrated with Ni-NTA binding buffer. The loaded column was rinsed with ten-column volumes of binding buffer, followed by ten-column volumes of wash buffer (respective Ni-NTA binding buffer with 30 mM imidazole), and finally, the bound recombinant protein was subjected to a gradient elution to 100 % buffer B (respective Ni-NTA binding buffer with 500 mM imidazole). Fractions containing recombinant protein were pooled after SDS-PAGE analysis and incubated with TEV protease overnight at 4 °C while dialyzing against the Ni-NTA binding buffer without imidazole. The next day inverse Ni-NTA purification was performed.

After inverse Ni-NTA purification, depending upon the purity of the protein performed, the final refinement was achieved through either size-exclusion chromatography (for cTnC, cTn IC binary, or cTn ITC tertiary complex) or further affinity

purification through Heparin chromatography (cTnT) or affinity purification through Heparin chromatography and size-exclusion chromatography (for cTnI) in their respective buffers. Peak fractions of the SEC column were analyzed on SDS-PAGE for their purity before pooling the fractions. The pooled fractions of cTnC (18.6 kDa), cTnI (24.2 kDa), the binary complex cTn IC (42.8 kDa), or of the tertiary complex cTn ITC (78.3 kDa) were concentrated using either Viva spin (GE) concentrators with 10 kDa cut-off or with the regenerated cellulose (Millipore) concentrators with 10 kDa cut-off. The concentrations of the proteins were determined at the nanodrop, and smaller aliquots of the concentrated protein were stored at -80 °C. Phosphorylation of the cTnI was performed with the recombinant full-length PKAc (protein kinase A, isoform c) enzyme from *C. griseus* expressed and purified in-house [16]. Purified cTnI was incubated with and without PKAc, in the presence of ATP and PKA NEBuffer™ for Protein Kinases, which provided the divalent cations. The phosphorylation reactions were performed at 37 °C, for two hours. After the assay time, the samples were frozen until further analysis by MS or ³¹P-NMR-based characterization. NMR experiments were performed at a measuring temperature of 298 K on a Bruker 950 MHz spectrometer equipped with a cryo-TXI-HCN probe. NMR samples were prepared with 5 % D₂O to lock the spectrometers, and 3-(Trimethylsilyl)-1-propane sulfonic acid sodium salt (DSS; 10 μM) was used as an internal standard for spectral referencing. NMR spectra were processed and analyzed in Topspin version 3.1 (Bruker Biospin). Sample conditions: 0.1-0.15 mM protein in 50 mM Tris pH 8.2, 200 mM NaCl, 5 mM CaCl₂, 2 mM TCEP. ³¹P-NMR spectra were measured at 298 K on a Bruker 500 MHz spectrometer equipped with a prodigy BBO-500 S1 probe. Sample conditions: 0.1 mM protein in NEBuffer™ for Protein Kinases (50 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT, 0.01 % Brij 35, pH 7.5).

2.4.6. MALDI-TOF-MS of expressed troponins

C4 ZipTips from Merck Millipore (10 μL) were used to desalt the samples according to the manufacturer's protocol. Proteins were eluted from the tip with 2.5 μL of CHCA MALDI matrix solution (10 mg/mL, 69.9 % purified water, 30 % acetonitrile, 0.1 % trifluoroacetic acid) directly on a spot of the MALDI target. BSA was used as a relative mass calibrator with an expected mass of 66.4 kDa.

2.4.7. Calibration of cTnI by sandwich ELISA

For the quantification of the expressed cTnI, a sandwich ELISA was used. 100 μL of the secondary capture antibody, goat anti-mouse IgG (2 μg/mL in PBS, pH 7.4), was incubated in each well of a clear, high-binding microtiter plate (MTP) overnight at 4 °C. The MTP was subsequently washed twice with 250 μL PBS and incubated for one hour, at room temperature, with 100 μL of capture antibody (2 μg/mL of monoclonal mouse anti-cTnI IgG, clone 9707, in PBS, pH 7.4, Medix Biochemica). Next, the MTP was washed three times with PBS containing 0.05 vol% of Tween 20 by an automated plate washer, and the wells were blocked with 250 μL PlateBlock (Candor Bioscience GmbH) for one hour at room temperature. After another wash with PBST, 100 μL of the cTnI calibrator (recombinant cTnI from HyTest Ltd.) and the expressed cTnI (both diluted in LowCross-Buffer, Candor Bioscience GmbH) were pipetted and incubated for one hour at room temperature. After a further washing step with PBST, the primary detection antibody was added. The polyclonal goat anti-cTnI IgG (HyTest Ltd.) was biotinylated by the following protocol: 2 μL of freshly prepared NHS-PEG12-Biotin (4 mg/mL in ultra-pure water) were added to 10 μL of 2 mg/mL antibody, mixed gently, and incubated overnight at 4 °C. The biotin-conjugated primary detection antibody was diluted 1:10,000 in LowCrossBuffer (Candor Bioscience GmbH), and 100 μL were transferred into the microtiter plate. After a one-hour incubation at room temperature, the plate was washed with PBST and incubated for 0.5 h with 100 μL of Neutravidin-HRP in the dark (1:30,000 dilution in LowCrossBuffer). Finally, the MTP was washed and incubated with 100 μL of TMB solution (Seramun Blau fast2). After approx. 10 min, a sufficient color change occurred,

and the reaction was stopped with 100 μ L of 0.25 M sulfuric acid. The absorbance was detected with an Epoch2 MTP photometer at 450 and 620 nm. The measured absorbance of the recombinant cTnI was used to establish a calibration for the biosensor measurements.

2.5. Antibody Labeling and Functionality Test

In order to remove preservatives and other potentially interfering compounds, 10 μ L of cTnI antibody (clone 9707) with a concentration of 2.6 mg/mL (stated by the supplier) was purified with a Zeba micro spin desalting column (at a 7 kDa molecular cut-off (Thermo Scientific)), equilibrated with PBS pH 7.4, and an additional added stacker volume of 3 μ L PBS pH 7.4. For the enzyme conjugation, a commercially available HRP Conjugation Kit-Lightning-Link (Abcam) was used. Therefore, 13 μ L of the purified antibody was added to 10 μ g pre-activated HRP and performed as specified by the manufacturer. An approximately two-fold molar excess of activated HRP was used relative to the antibody. The ability of the antibody-HRP conjugate to bind to the recombinant cTnI, as well as to the synthesized peptide-BSA conjugate, was tested by two approaches: A direct ELISA with peptide-BSA conjugate and a competitive ELISA with recombinant (rec.) cTnI.

For the direct ELISA and the test of the synthesized 9707-HRP conjugate, 100 μ L of the peptide-BSA conjugate in PBS (pH 7.4, 0.8 μ g/mL) was incubated on a high-binding MTP overnight at 4 $^{\circ}$ C and 750 rpm. After washing three times with PBST (pH 7.4 and 0.05 % Tween20), further mentioned as a washing cycle, the wells were blocked with 300 μ L of 1 % BSA in PBST pH 7.4 and incubated for two hours. After a washing cycle, 100 μ L 9707-HRP was diluted differently in PBST-BSA (0.05 % of Tween 20, 0.2% of BSA, pH 7.4) and transferred into the wells and shaken for 1 h at 750 rpm. After the final washing cycle, 100 μ L TMB substrate was added and stopped with 0.25 M sulfuric acid.

The second approach involved a competition between the recombinant cTnI analyte and the peptide-BSA conjugate. Therefore, the peptide-BSA conjugate was immobilized on a transparent high-binding MTP (100 μ L per well with 8 μ g/mL). After overnight incubation at 4 $^{\circ}$ C, followed by a washing cycle, the plate was blocked with 300 μ L of 2 % BSA in PBS (pH 7.4) for two hours at 750 rpm. After another washing cycle, 50 μ L of different concentrations of the recombinant cTnI were added to each well, followed by 50 μ L of 1:50,000 diluted 9707 monoclonal antibodies (all in PBST, 0.1 % Tween20, BSA 0.1 %), pH 7.4). Next, the plate was shaken for one hour at 750 rpm, followed by a washing cycle. Next, 100 μ L of peroxidase-conjugated goat anti-mouse IgG (1:50,000 diluted) was added and incubated for one hour at room temperature and 750 rpm. Finally, the MTP was washed again, and 100 μ L TMB substrate was transferred and stopped with 0.25 M sulfuric acid after approximately 10 minutes. The absorbance was recorded with an Epoch2 photometer at 450 and 620 nm.

2.6. Chemiluminescence Detection

The optical detection of the chemiluminescence is based on the commercially available camera QHY174M-GPS from QHYCCD (Beijing, China) with a CMOS sensor and a custom-designed flow cell, which is connected via T2 thread (M42x0.75). The camera uses the highly sensitive Sony IMX174 front-side illuminated CMOS sensor (11.25x7.03 mm in size) with 2.3 megapixels (19,20x1,200 pixels) and a pixel pitch of 5.86 μ m. The camera and the connected flow cell are shielded by a black enclosure to remove the influence of ambient light.

The flow cell was designed and precision manufactured in-house and consisted of POM; see Figure 3. The cover plate and the enclosure were additively manufactured and consisted of acrylonitrile butadiene styrene (ABS), retaining the planoconvex lens (12 mm focal length) with a diameter of 9 mm via O-ring. Both were mounted and sealed with five DIN 7991 - M3x8 screws.

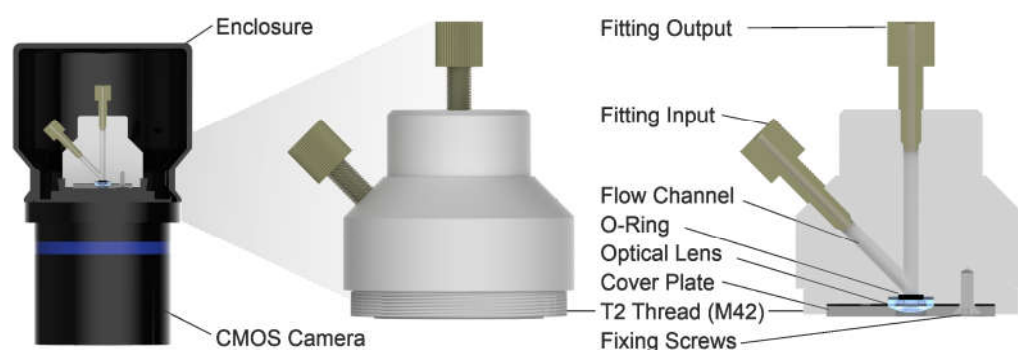


Figure 3. Flow cell design for chemiluminescent detection. The flow cell was manufactured from POM, and the enclosure was made from ABS.

Through a little gap between the CMOS camera and the enclosure (not shown), microfluidic input and output tubing are guided to enter and exit the flow cell through the enclosure. The samples, including the analyte and HRP-labeled antibodies, which have been pre-mixed with the chemiluminescence substrate, are pumped through the cell. The chemiluminescence is based on a fast peroxidase-catalyzed reaction between luminol and hydrogen peroxide and the development of an activated intermediate. Once this product decays to the ground state, it emits light with an approx. wavelength of 450 nm, and the released photons can be detected. The CMOS sensor receives the collected light from the flow channel focused by the planoconvex optical lens and an additional planoconcave optical lens (9 mm diameter and 12 mm focal length), which is located on top of the CMOS sensor glass cover with a few microliters of immersion oil from Zeiss (see Figure S10).

2.7. Fluidic Setup and Measurements

Initially, for equilibration and to remove all air bubbles inside the tubings and the flow cell, the whole microfluidic setup was flushed with filtered (0.2 μm) and degassed running buffer (PBS with 0.05 % of Tween 20 and 0.1 % of BSA). Subsequently, the running buffer and chemiluminescence substrate (SuperSignal West Pico Plus) were injected with a high-precision Fusion 4000X dual syringe pump from Chemyx. The buffer flow was directed through a six-way valve, which was used to inject 500 μl samples via a sample loop to the monolithic affinity column (see Figure 4). After passing the monolithic column, the chemiluminescence substrate was blended in the sample flow with a microfluidic pearl-chain mixer, as shown in Figure S9. In this process, the sample flow was diluted and mixed 1:1 with the chemiluminescence substrate and incubated for around 11 min in an incubation loop. The chemiluminescence signal was detected via a CMOS sensor connected to the flow cell. The exit flow was collected in a waste beaker and discarded.

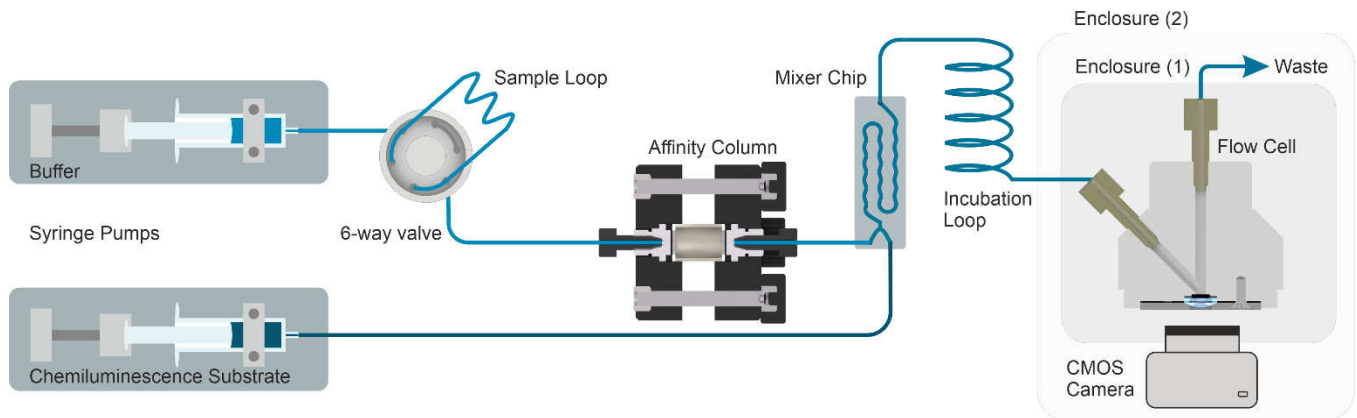


Figure 4. Microfluidic setup for the detection of cardiac troponin I (cTnI). The sample injection was performed by a 6-way valve and a continuous flow of running buffer and chemiluminescence substrate. After passing the peptide-BSA affinity column, the sample and substrate were mixed 1:1 inside a mixer chip, followed by an incubation loop for preincubation and the flow cell for optical detection.

For the detection, a sequence of single images was captured by the CMOS camera with an exposure time of 15 seconds each. The sensor was cooled to -5°C during the whole measurement to improve the noise performance further. Due to the temperature-dependent nature of the enzymatic reaction, the temperature inside and outside the enclosure was closely monitored continuously by sensors.

2.8. Data analysis

To capture the sensor data, the Software SharpCap 3.2 was used. Images were taken in fifteen seconds intervals and saved as 12-bit FITC-raw files. The gain was set to zero, 2x2 pixel binning was selected (final image size 960x600 px), and the sensor temperature was set to -5°C . Image evaluation was performed by a custom Python script (see script.py in supplementary materials) with a fixed region of interest (ROI) of 640x600 px (see Figure S11) and exported as text (txt) files. These results were evaluated using Origin 2020 (9.7.0.188). Based on the determined intensities, the areas of the sample peaks were integrated and plotted graphically.

3. Results

3.1. Troponin Peptide-BSA Conjugation and Characterization

For the conjugation of the peptide derived from a troponin sequence to BSA, the bifunctional crosslinker SBAP was chosen. In the first step, SBAP was conjugated to some of the primary amines of BSA (lysine side chains) via NHS ester reaction (SBAP-BSA). In the second step, after adding Peptide188-199, the free bromoacetyl groups on the BSA reacted with the N-terminal cysteines of the peptide. The conjugate (Pep188-199-BSA) was analyzed by MALDI-TOF MS. Approximately twenty molecules of SBAP, and five peptides per BSA were bound on average (Figure 5).

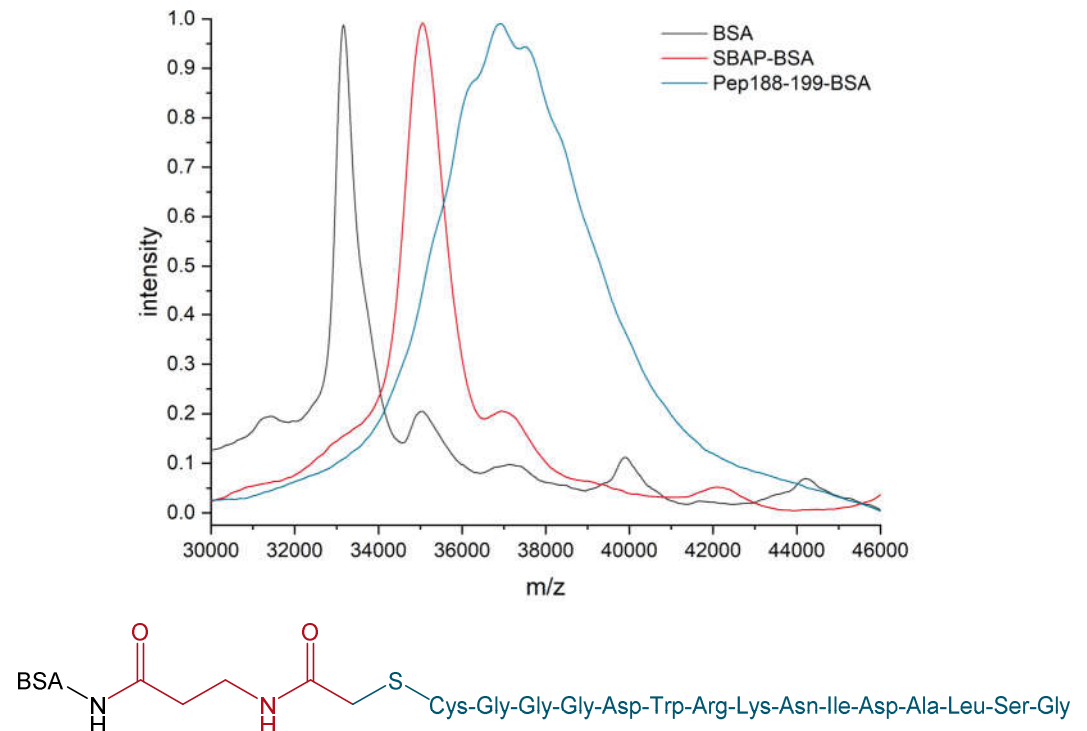


Figure 5. MALDI-TOF-MS of doubly charged species $[M+H]^{2+}$ of BSA (black), BSA-SBAP (red), and final Pep188-199-BSA conjugate (blue). For the coupling density of the final BSA-peptide conjugate, the spectrum was fitted non-linear with a Lorentz-fit and Levenberg Marquardt iteration algorithm (fit not shown), and the maximum was used for the calculation. Below, a schematic structure of the conjugate is shown. The C-Terminus of the peptide is amidated. Due to the shift in the spectrum towards the higher mass range, an average of about five peptides per BSA could be determined.

3.2. Expression of cTnI in *E. coli* and adjustment of the resulting stock solution by ELISA

3.2.1 Expression of human cardiac troponins in *E. coli*

The expression of human troponins was performed in the well-established bacterial expression system due to its ease of handling and its relatively cost-effective and proven strategies for uniform isotopic labeling for NMR spectroscopic studies. Individual subunits of the cTn were subcloned into the in-house modified pET32 inducible expression vector (see Figure S1), which facilitated the addition of a TEV protease cleavable N-terminal His tag to the target protein in *E. coli* (see Figure S2). At first, each troponin subunit was transformed into T7-express competent cells. Then, test expressions were performed with the transformants on a small scale for which we could observe overexpression of the individual troponins, respectively. However, the expression levels were not consistent upon repetition. Therefore, each troponin subunit was expressed in different competent cells, such as BL21 (DE3) or T7 express or T7 express lysY/Iq, to achieve tight regulation on the basal expression of the target gene. The test expressions of these various transformants performed in small volumes showed that the individual expression of all three troponins was highest in the T7 express lysY/Iq cells with

reproducible results. Henceforth, we used T7 express lysY/Iq cells for all scale-up expressions.

3.2.2 Purification and characterization of recombinant cardiac troponins

We report here the native purification conditions for the individual subunits of human cTn full-length proteins. The purification of cTn's was performed from the cell pellet obtained by the overnight expression performed at 20 °C after cold shock and induction with 1 mM IPTG. Surprisingly, the protein yield after affinity chromatography was significantly lower for cTnI and cTnT, contrary to the test expression yields of the respective proteins. Therefore, cTnI and cTnT proteins were expressed at 37 °C for four to five hours, notably improving expression yields. For cTnI purification after the first Ni-NTA affinity chromatography, we observed precipitation of the protein upon removal of the fusion tag (and dialysis to remove imidazole) due to its hydrophobic nature. Therefore, we supplemented the buffers with arginine and glutamic acid, which prevented protein aggregation and precipitation to a larger extent [20]. We used similar buffer conditions for cTnT purification that were unnecessary for cTnC protein purification. In the protein purification process, we could achieve active cTnI protein in native conditions to ~ 1-2 mg, active cTnC protein in native conditions to ~ 20 mg, and cTnT to ~ 10 mg per liter culture. The final products have been further characterized by SDS-PAGE, and bands appear according to the molecular size before and after the cleavage with TEV protease, shown in Figure S2.

For biosensor development, recombinantly produced cTnI will be used. In order to assess that the recombinant cTnI is folded correctly, we performed size-exclusion chromatography (SEC) and 2D NMR analysis using ¹⁵N-isotope enriched protein. Formation of the binary (cTn IC) and ternary (cTn ITC), as confirmed by the SEC profile (shown in Figure S3A), suggests that the protein is active and well folded. Further, the successful formation of the cTn IC complex was investigated by recording 2D NMR, ¹H, ¹⁵N-HSQC spectrum of the proteins. An overlay of the ¹H, ¹⁵N-HSQC spectra of free cTnI and the cTn IC complex revealed significant changes in the amide chemical shifts indicating complex formation (Figure S3B and C).

3.2.3 Calibration of the stock solution of recombinant cTnI by ELISA

Recombinantly produced cTnI expressed and purified from *E. coli* was used for the biosensor measurements. A calibration with commercial recombinant cTnI was performed, and a sandwich-ELISA was established. Subsequently, the concentration of the stock solution could be determined and used for the measurements (Figure 6).

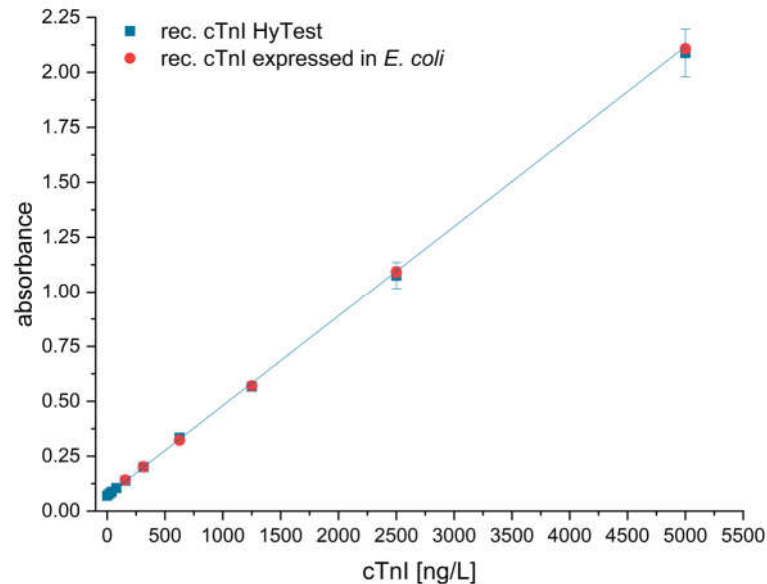


Figure 6. Quantification of cTnI expressed in *E. coli* by using commercially available cTnI (Hytest 8RTI7), which was used to calibrate the sandwich ELISA, as recommended by the supplier [21].

3.3. Performance of Peptide-BSA Column and Blank Measurement

cTnI samples were analyzed with a direct and competitive indirect ELISA, shown in Figures S5 A and B, to confirm the functionality of the peptide-BSA conjugate and the specificity of the antibody-HRP conjugate. Optimal CMOS sensor parameters, like active cooling and incubation times for the chemiluminescence signal development, have previously been tested and are shown in Figures S6 and S7.

The performance of the functionalized peptide-BSA affinity column and the 9707-HRP conjugate were tested by injecting the highly diluted reagent (1:60,000 dilution). The relative chemiluminescence was measured with and without an inserted affinity column, as shown in Figure 7.

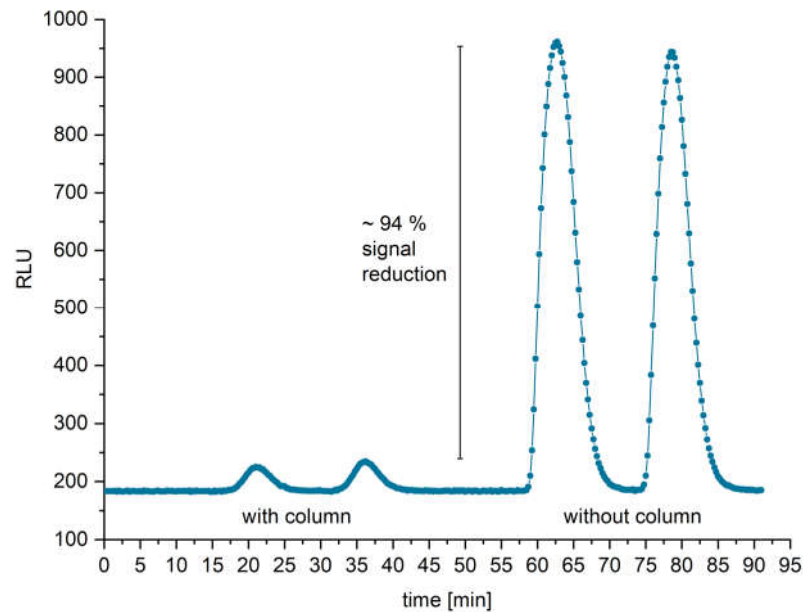


Figure 7. Performance test of the functionalized Pep188-199-BSA column and the antibody-HRP conjugate (clone 9707). Injection of blank samples with and without affinity column resulted in a signal reduction of around 94 % by applying a flow rate of 0.2 mL/min for each syringe pump. The signal reduction is an indication of conjugate purity and antibody activity.

The signal difference provides information about the binding interaction of the antibody with the affinity column. The signal was reduced by up to 94 % by inserting the affinity column. This means that this fraction of the antibody-HRP conjugate was bound to the peptide-BSA column. 6% of the conjugate was not trapped, which might be caused by inactive bioconjugates or non-conjugated HRP and might be further reduced by additional conjugate purification steps. Subsequently, the antibody-HRP conjugate was injected six times into the column, shown in Figure 8. A stable baseline was obtained, and the peak area of each blank was integrated, resulting in a relative standard deviation of 4.4 %.

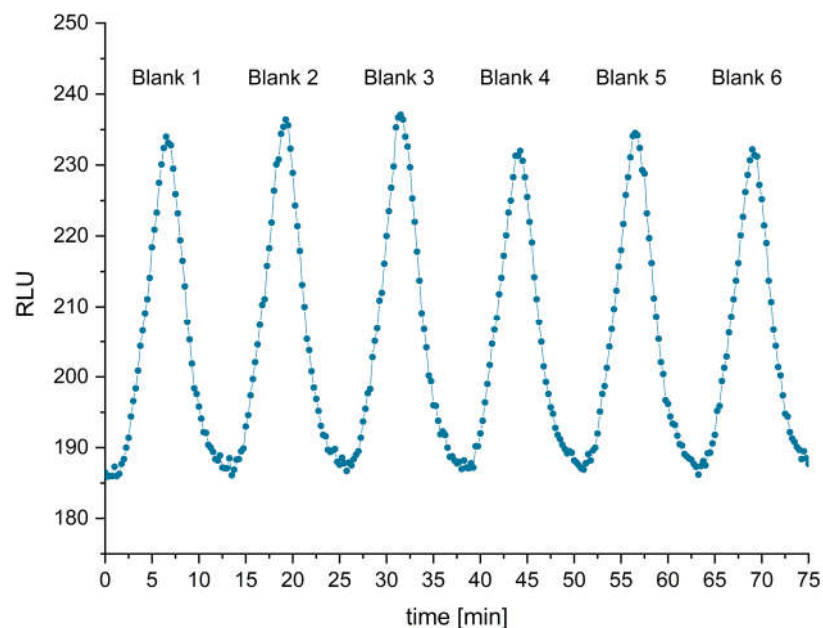


Figure 8. The injection of a sample series with six blanks achieved a standard deviation of 4.4 % using peak integration.

3.4 cTnI Measurement

First, the dynamic range of the experimental biosensor setup was examined by the injection of different concentrations of recombinant cTnI diluted in PBST-BSA buffer (0 to 64 µg/L), shown in Figure 9.

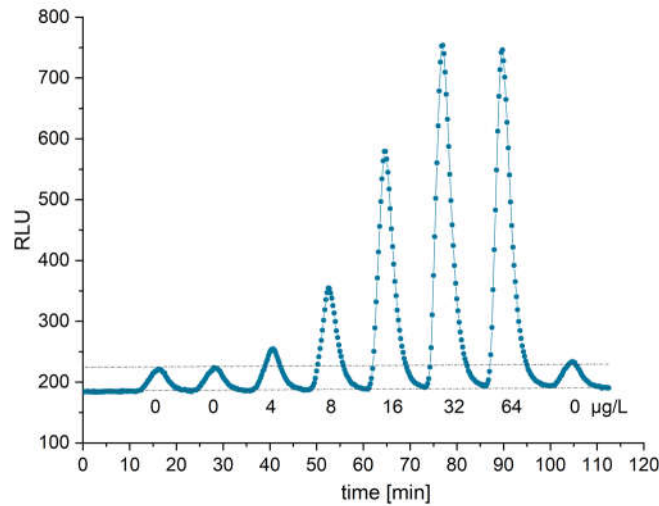


Figure 9. Measurement of recombinant cTnI in a concentration range between 0 and 64 µg/L.

Over the whole measurement time, constant baseline and blank values were obtained. The chemiluminescence signal increased asymptotically with higher analyte concentrations up to 32 µg/mL and reached saturation. The lowest cTnI concentration, which resulted in a positive signal, was 4 µg/L.

3.5 Test of Specificity (Cross-Reactivity)

To ensure the antibody specificity and to rule out interference of the analyte storage buffer, different samples were tested in duplicates: cTnC, cTnT, cTnI, and the sample buffer. In addition, the blank (running buffer) was also measured as a reference (see Figure 10).

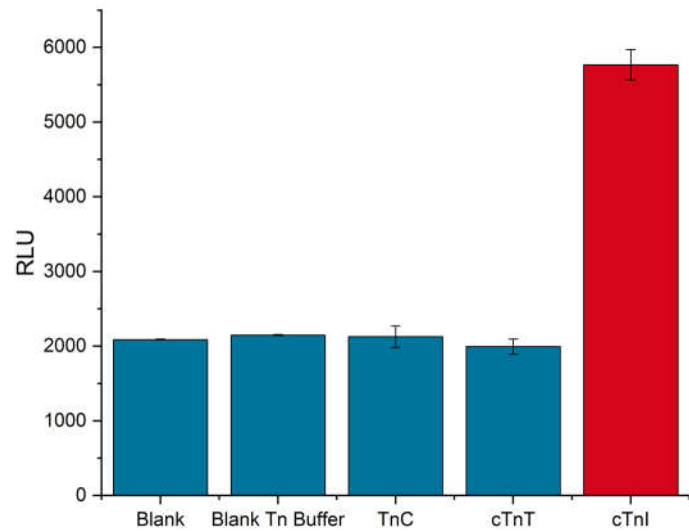


Figure 10. Antibody specificity test against cTnI: The biosensor detected cTnI (red column). No cross-reactivity with cTnT, cTnC, or any influence of the sample buffer was observed. Chemiluminescence was quantified by integration of the peak area. Error bars represent standard deviations.

For the cross-reactivity test of the biosensor, each cTn species was diluted to 16 $\mu\text{g/L}$. No interference with the sample buffer was observed. For all samples but the cTnI, chemiluminescence signals at the background level were obtained, the same as for the negative controls.

3.6 Linearity of cTnI Measurement in PBST-BSA Running Buffer

The biosensor's linearity and limit of detection (LOD) were investigated. Therefore, various concentrations of cTnI were measured, ranging from 0 to 16 $\mu\text{g/L}$, as shown in Figure 11.

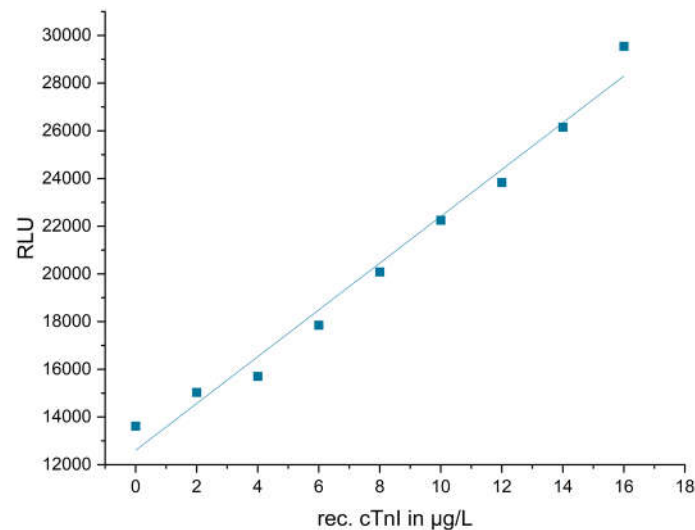


Figure 11. Calibration with a linear fit for troponin I in the range of 0, 2, 4, 6, 8, 10, 12, 14, 16 $\mu\text{g/L}$ and appropriate calibration curve after peak integration. Sensor data can be found in Figure S12.

The LOD of 2.8 $\mu\text{g/L}$ was calculated based on three times the blank measurements' standard deviation (4.4 %) (Figure 8).

3.7 cTnI Measurement in Human Plasma and Serum

Finally, the biosensor was tested with relevant blood matrices. Recombinant cTnI was spiked into human plasma with defined Troponin concentration and human serum. Blood plasma is not depleted of cTnI and was additionally spiked with it. However, this is considered in the blank measurements of the plasma, shown in Figure 12. On the other

hand, the blood serum from HyTest is cTnI depleted. The samples were diluted forty-fold with the running buffer to reduce matrix effects.

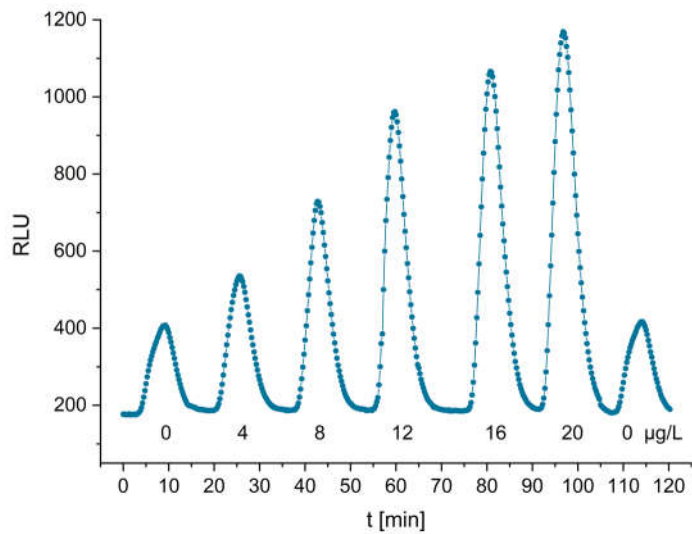


Figure 12. Measurement of human plasma samples spiked with rec. cTnI between 0 and 20 µg/L diluted 1:40 in running buffer.

The chemiluminescence intensity increases with higher concentrations of cTnI in a linear range between 0 and 800 µg/L (see Figure 13). LODs of 40 µg/L for plasma and 140 µg/L for serum were obtained based on the blank value series with a standard deviation of 4.4 %, shown in Figure 8.

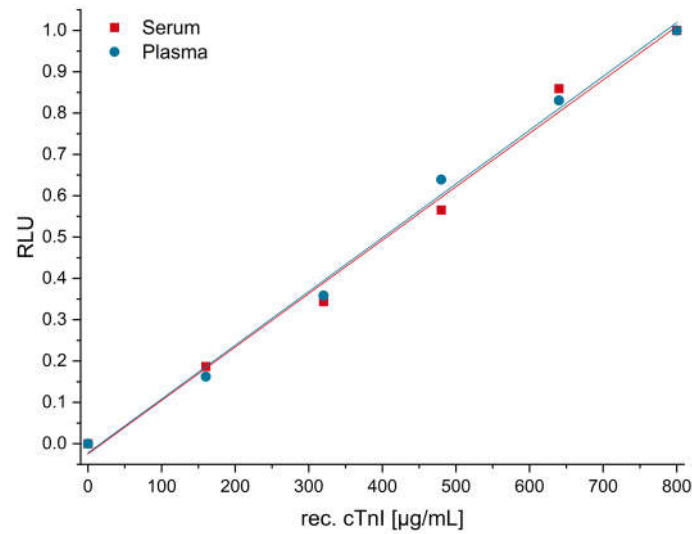


Figure 13. Linearity of the cTnI detection in spiked human plasma and purified human serum. The samples were diluted 1:40 in the running buffer. In contrast to Fig. 12, this dilution factor is considered for the calculation of the LODs of 40 µg/L (plasma) and 140 µg/L (serum), respectively.

4. Discussion and Conclusion

In this work, a proof-of-concept of a chemiluminescence-based biosensor for the detection of cTnI is presented. The samples are mixed with an antibody-HRP conjugate, measured quasi-continuously via a microfluidic setup, and fitted to a CMOS-based detection system. The assay takes place on an affinity column and allows the detection of cTnI-blocked antibodies after mixing and incubating with chemiluminescence substrate in a mixer chip. Unfortunately, fluorescence-based detection is barely suitable for high-sensitivity detection since blood serum or plasma usually show heavy autofluorescence even in the far-red spectral range, leading to a high background (Figure S13). In order to overcome this limitation, a novel flow cell prototype was designed, which allows the near real-time detection of chemiluminescence. The enzyme horseradish peroxidase (HRP) was covalently linked to a target-specific antibody and used with a luminol-based chemiluminescence substrate for signal generation. HRP reacts with H_2O_2 and luminol, resulting in efficient light emission (~ 450 nm). Unfortunately, blood serum and plasma seem to suppress luminol chemiluminescence to some degree [22-24]; hence, some dilution of the samples is necessary.

Furthermore, the obtained LODs need to be further improved to achieve a comparable sensitivity with high-sensitivity immunoassays [25,26]. Based on the literature, it is known that this biosensor format would benefit from monovalent binders, such as Fab or nanobodies [27-30]. A binder of the highest possible affinity would be desirable in all cases. Those might be obtained by affinity maturation of existing recombinant antibodies [31] or advanced library selection methods [32]. Also, different enzymes should be explored which are perfectly compatible with the plasma or serum matrix and might allow for a faster reaction. Finally, a much lower sample dilution would highly contribute to better sensitivity.

Preparing and characterizing troponins as reference materials is the crucial step toward traceable diagnostic procedures. All three individual subunits of the human cTn complex were successfully expressed in *E.coli*. cTnI, cTnT, and cTnC were purified near homogeneity and used in cross-reactivity tests. Further, the recombinantly produced cTnI and the cTnI purchased from Hytest behave very similarly in the ELISA, confirming that the proteins are correctly folded and active. In the next development step, continuous plasma sampling could be envisaged. This continuous plasma monitoring would be a big leap in abandoning the one-time measurements, whether a laboratory-based system or a quick test.

Supplementary Materials: Figure S1: Human cardiac troponin constructs and expression. Figure S2: SDS-PAGE of all three troponin subunits (cTnI, cTnT, and cTnC). Figure S3: Human cardiac troponin binary and ternary complexes. Figure S4: MALDI-TOF-MS of rec. cTnI, cTnT, and cTnC expressed in *E. coli* and their expected mass: 24.4 kDa for cTnI, 35.9 kDa for cTnT, and 18.6 kDa for TnC. Figure S5: 9707-HRP antibody binding against BSA-Pep188-199 conjugate (A) and 9707 antibody competition between the BSA-Pep188-199 conjugated and the expressed rec. cTnI (B). Figure S6: Investigation of the noise behavior of the CMOS under various conditions. Figure S7: Relative luminescence unit (RLU) measurement of three different sample matrices (PBST-BSA, human serum 1:40, and human plasma 1:40). Figure S8: Drawings of the flow cell made by subtractive manufacturing and the enclosure. Figure S9: Drawing of the pearl-chain mixer chip (658) ordered from microfluidic ChipShop. Figure S10: Top view QHY 174 mono CMOS with a planoconcave lens positioned on top of the protective glass of the separate CMOS chamber. Figure S11: Images of the CMOS with 2x2 pixel binning resulting in an image size of 900x600 px. Figure S12: Calibration of recombinant Troponin I in PBST-BSA in the lower concentration range. Figure S13: Autofluorescence measurement of blood plasma with the previously described biosensor setup. Table S1: Preparation of the BSA-Peptide-188-199 column.

Author Contributions: Conceptualization, M.G.W. and R.T.; methodology, M.P., and M.G.W.; software, M.P.; validation, R.T.; formal analysis, R.T., and M.G.W.; investigation, R.T., resources, M.G.W.; protein expression and purification: S.G., S.S., K.S., C.R., and H.S.; technical drawings and prototyping: B.R., R.T., and M.G.W.; protein analysis and characterization: S.G., S.S., R.T., K.S., C.R. and H.S., data curation, R.T.; writing—original draft preparation, R.T., C.S., and M.G.W.; writing—

review and editing, R.T. M.P., C.S., S.S., and M.G.W.; visualization, R.T. and M.P.; supervision, M.G.W.; project administration, C.S., and M.G.W. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Technical Drawing of the flow cell: drawing.pdf; Python script for data evaluation: script.py, including corresponding txt-files: hotpixels_960_600.txt and roi_region_x_y.txt. Furthermore, eleven test FITC files can be found in the Supplementary Materials.

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Conflicts of Interest: The authors declare no conflict of interest.

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