Published in final edited form as:

Proc SPIE Int Soc Opt Eng. 2020 February ; 11251: . doi:10.1117/12.2546979.

Enhancement of label-free biosensing of cardiac troponin I

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Abstract

The detection of cardiac troponin I (cTnI) is clinically used to monitor myocardial infarctions (MI) and other heart diseases. The development of highly sensitive detection assays for cTnI is needed for the efficient diagnosis and monitoring of cTnI levels. Traditionally, enzyme-based immunoassays have been used for the detection of cTnI. However, the use of label-free sensing techniques have the advantage of potentially higher speed and lower cost for the assays. We previously reported a Photonic Crystal-Total Internal Reflection (PC-TIR) biosensor for label-free quantification of cTnI. To further improve on this, we present a comparative study between an antibody based PC-TIR sensor that relies on recombinant protein G (RPG) for the proper orientation of anti-cTnI antibodies, and an aptamer-based PC-TIR sensor for improved sensitivity and performance. Both assays relied on the use of polyethylene glycol (PEG) linkers to facilitate the modification of the sensor surfaces with biorecognition elements and to provide fluidity of the sensing surface. The aptamer-based PC-TIR sensor was successfully able to detect 0.1 ng/mL of cTnI. For the antibody-based PC-TIR sensor, the combination of the fluidity of the PEG and the increased number of active antibodies allowed for an improvement in assay sensitivity with a low detection limit of 0.01 ng/mL. The developed assays showed good performance and potential to be applied for the detection of cTnI levels in clinical samples upon further development.

Keywords

Label-Free; Biosensing; Cardiac Troponin I; Photonic Crystal-Total Internal Reflection; Myocardial Infarction; Aptamer

1. Introduction

Myocardial infarction (MI), or a heart attack, occurs during periods of blood deprivation in the myocardium and can lead to necrosis in these areas. MI has a high prevalence in the United States (US) and is a major contributing factor to heart disease; being the leading cause of death in 2017 [1]. MI led to hospitalizations in almost 200,00 adults in the US between 2009 and 2010 [2], which does not include the large number of heart attacks that go undetected. Due to the small amount of time between the onset of MI and the beginning of cell necrosis, as short as 20 minutes [3], quick detection of a heart attack is critical to improving patient outcomes.

Upon cell death or injury, cell membranes break down and lead to the release of compounds and macro-molecules contained inside of the cells. The released molecules can serve as biomarkers for the effective diagnosis and screening of MI. Cardiac troponin I is one of the most widely used biomarkers for detection of MI, due to its high specificity to cardiac tissue [4]. Small elevations in cTnI at levels as low as 0.1 ng/mL can signify the occurrence of a MI. [9] Once a patient has been admitted into the hospital, blood tests over time are used to determine whether or not significant enough changes in these values have occurred to give a diagnosis of MI. Improving the speed and ease at which blood tests are run while maintaining the ability to detect clinically significant ranges of cTnI is important for improving outcomes of MI.

Since early diagnosis is an important variable in determining the outcome of MI, many different tests have been developed to accurately detect the presence of cardiac biomarkers in blood. Currently, enzyme linked immunosorbent assay (ELISA), surface plasma resonance (SPR), and high-performance liquid chromatography (HPLC) are widely used in clinical settings to identify these biomarkers [5], however, there are drawbacks with each method. These tests are limited by either their inability to detect cTnI at the point-of-care quickly, or by their high detection limits and low sensitivity. Fluorescence based tests provide much higher sensitivities and can detect smaller amounts of cTnI but are limited by the longer test times due to sample alterations needed to perform the tests [7]. There are many different label-free sensing methods that have been developed but are all limited by either their sensitivity or cost ([10]; [11]; [12]; [13]; [15]; [16]; [17]). Improving the sensitivity of label-free detection represents the opportunity to make a significant advancement in the diagnosis and treatment of MI.

An alternate platform is the Photonic Crystal Total-Internal-Reflection (PC-TIR) sensor, which was developed to provide a base for high sensitivity, label-free detection [17]. The sensor is based on the principle of a microcavity structure rather than a traditional closed cavity. Total internal reflection is used to create a virtual mirror image of the sensor structure. The open cavity allows for an easily functionalized surface with high sensitivity to the composition of the material on the sensor surface. Previous testing done with photonic crystal sensors has proven its capabilities of analyte detection in real time ([14]; [17]; [19]; [20]; [21]; [23]; [24]). In tests specific to cTnI, concentrations as low as 0.1 ng/mL were quantifiable using the PC-TIR sensor [22]. While this is in the clinically significant range, it does leave further room for improvements on efficiency and sensitivity to lower concentrations to achieve results similar to fluorescence-based tests. Further, matrix effect from real samples such as human plasma can increase the effective detection limits of the assay. It is therefore important to develop assay that are highly sensitive to overcome these limitations.

In this light, this paper presents two approaches to improving the sensitivity and performance of cTnI detection on the PC-TIR sensor. Firstly, an antibody-based PC-TIR sensor is developed by utilizing recombinant protein-G (RPG) to effectively orientate anti-cTnI antibodies on the PC-TIR sensor surface, thereby increasing the cTnI capturing efficiency of the antibodies. The manuscript also explores for the first time, the use of an anti-cTnI DNA aptamer to serve as biorecognition elements for the development of an

aptamer-based PC-TIR sensor. Aptamers are single stranded nucleic acid oligomers that are developed through a process termed Systematic Evolution of Ligands by Exponential Enrichment (SELEX) to bind target molecules with high selectivity and sensitivity. Aptamers have the advantage of being easy and cheap to synthesize, thereby reducing sensor development costs. Furthermore, aptamers are more thermally stable than antibodies and can therefore be used without refrigeration. Both sensing approaches utilize high molecular weight polyethylene glycol (PEG) molecules as linkers. The PEG allows for greater fluidity of the functionalized surface and lead to increased binding between analyte and the respective biorecognition elements.

2. Methods

2.1: Fabrication of microfluidic channels

A 5-channel microfluidic system was developed in order to deliver chemicals to the sensor surface for functionalization. A clean silicon wafer was placed onto a hotplate at 200°C for 15 minutes to allow dehydration of the surface followed by placement in an O₂ plasma cleaner for 45 seconds. Negative photoresist, SU-2025 (MicroChem) was then spin coated onto the wafer surface at 500 rpm for 30 s followed by 1200 rpm for 1 min resulting a coating thickness of approximately 80 µm. The photomask with 5 channels, each with a width of 400 µm and length of 6 mm, was placed over the coated sensor and allowed to cure under UV light for 18 s. The wafer then underwent a post-exposure bake at 95°C for 5 min and was then placed into SU8 developer (MicroChem) for 10 min. The wafer was then cleaned leaving behind the channel mold. To replicate the channels, PDMS (Sylgard184, Dow Corning) was mixed with a ratio of 10:1, base to curing agent. The mixture was degassed in a desiccator for 30 min and poured over the top of the master mold. Curing took place at 100°C for ~30 min. A newly fabricated PC-TIR sensor and the PDMS mold were placed into a plasma cleaner (Harrick Plasma) for 45 s, which oxidized the PDMS and created silanol groups on the sensor surface. The PDMS was then stuck onto the sensor surface where it formed stable covalent Si-O-Si bonds. The sensor is then placed into position in the laser pathway as described in section 2.3 and connected to the pneumatic pump system described in section 2.2.

2.2: Functionalization of the sensor surface

The functionalization steps of the sensor were as shown in Fig. 1. A pneumatic pump system utilizing on-board air was developed for ease of use and consistent flow rates to deliver samples to the sensor surface. This was connected to the microchannel mold described in section 2.1 using polytetrafluorethylene tubing to minimize interaction of samples with the tubing. Silane-PEG-biotin (from Nanocs) molecules were first applied to the surface at a concentration of 5 mg/mL. This was followed by streptavidin, which was immobilized through its interaction with the biotin moiety located at the end of the PEG linker molecules. RPG-biotin was then introduced and was captured by the remaining active sites left on the streptavidin molecules. Anti-cTnI (from Abcam) was then applied, which bound to the RPG molecules with optimal orientation to facilitate an increase in active sites available for cTnI binding. SEA blocker is used to fill in gaps left on the sensor surface to improve specificity

by preventing nonspecific adsorption of molecules onto sensor surface. The sensor was then ready for target cTnI introduction and sensing.

For the alternate approach that utilized anti-cTnI aptamers [25] as biorecognition elements, the sensor surface was modified with Silane-PEG-COOH (Nanosoft Polymers) dissolved at a concentration of 5 mg/mL in 95% v/v ethanol. The silane groups of the Silane-PEG-COOH were covalently coupled to the sensor surface. The free carboxyl groups at the end of the Silane-PEG-COOH were then activated by treating them with a flow of EDC/NHS (9.6 : 5.4 mg) dissolved a milliliter of MES buffer (pH=4.7) (ThermoFisher Scientific). The activated carboxyl groups were covalently coupled to the amine groups of the anti-cTnI aptamer (Integrated DNA Technologies, Inc.) at a concentration of 250 μ M. Prior to coupling the aptamer onto the sensor surface, the aptamer solution was heated to 95°C for 5 mins and immediately snap-cooled on ice for at least 15 minutes. The rapid cooling facilitated the folding of the aptamer into a hairpin-like structure which enhanced its performance. The sensor surface was then ready for the testing of sample solution containing cTnI.

2.3: Cardiac Troponin I binding assays

The setup of the PC-TIR sensor is shown in Fig. 2. The working principle behind the setup is that the resonant angle of each channel appears as a dark line in an image on the CCD camera. As binding occurs on the sensor surface the dark line will shift according to the change, allowing each channel to operate and be monitored independently. This shift in dark line is then converted to a wavelength shift based on the calibration of the sensor. Calibration is done using a white light source at a fixed angle of 64° as opposed to the helium-neon laser. The resonant wavelength is monitored in this setup using a spectrometer (HR 4000, Ocean Optics). Using different concentrations of ethylene glycol, the resonant wavelength can be compared to the pixel shift of the dark line for the corresponding solution in order to determine the nanometers per pixel shift.

After completing functionalization of the sensor surface, cTnI (abcam) at varying concentrations (0.01 ng/mL to 10 ng/mL) was allowed to incubate on the sensor surface for 30 min. Ultrapure water was then flowed over the surface before and after the cTnI incubation, the difference in the position of the dark line was used to quantify the antibody-antigen binding. Accurate calculation of the dark line position is done by fitting a Lorentzian function to the image intensity profile.

3. Results and Discussion

3.1: Sensor Calibration

In order to calibrate the system, two different experimental setups were used to calculate the number of pixels corresponding to a specific shift in the resonant wavelength. Replacing the He-Ne laser used in Fig. 2 with a white light source at a fixed angle allowed the wavelength associated with the resonant frequency to be absorbed by the silicon layer in the microcavity. This produced a dip in the reflectance spectrum similar to the production of the dark line when using a probe laser. Using different concentrations of ethylene glycol with the two

different setups allowed for comparison between the wavelength and pixel shifts. The image in Fig. 3a shows the dark line shifts of the sensor when pure water was run through the channels. Fig 3b shows the shifts when ethylene glycol concentrations of 20, 15, 10, 5, and 0% weight in water, from top to bottom, was flowed through the channels. The corresponding pixel shifts are shown in Fig. 3c and 3d. These values are compared to the resonant wavelength shift found using a white light source and the same concentrations of ethylene glycol. The conversion factor between wavelength and dark line shift was experimentally determined to be 0.039 nm/pixel.

The binding graphs for the immobilization process are shown in Fig. 4. Each graph shows the initial baseline calculation in step 1 using distilled water, followed by the shift in real time of the respective sample. Step 3 in each graph shows the resultant shift back towards the baseline by flowing water. After the wash has stabilized the final resonant wavelength shift is calculated by determining the dark line shift in pixels between the stabilized values in steps 1 & 3, then converting to nanometers using the 0.039 nm/pixel conversion factor.

3.2: Cardiac Troponin I Binding Assays

After functionalization was completed by the respective methods, cTnI solution are applied with varying concentrations. The shift due to antigen binding is calculated by comparing relative the dark line positions between running ultrapure water through the sensor microchannels and running sample cTnI solutions.

The calculated shift in resonant wavelength due to antigen binding for each experiment is shown in Fig. 5. The RPG based functionalization method proved to push the detection limit of the PC-TIR sensor past the previously shown limit of 0.1 ng/mL. Flowing a cTnI solution of 0.01 ng/mL resulted in a resonant wavelength shift of 0.71 nm. In comparison, the aptamer-based functionalization method did not generate a detectable shift for the same cTnI solution at 0.01 ng/mL. Although the aptamer based assay showed lower sensitivity, the use of aptamers presents the advantages of not requiring refrigeration, and having lower cost and ease of production than antibodies.

Both methods of functionalization were able to successfully monitor the RI changes of the sensor surface as cTnI bound to the surface biorecognition elements. The aptamer-based measurement achieved the sufficient sensitivity with fewer functionalization steps. The RGP orientation of anti-cTnI antibodies in the antibody-based PC-TIR sensor proved that controlling the orientation of the antibodies can result in more efficient binding and thus higher assay sensitivity as demonstrated by the improved detection limit.

4. Conclusion

The PC-TIR biosensor provides an excellent platform for improving the capabilities of label-free detection. The open microcavity design allows for simple integration into a microfluidic system and easy functionalization processes. The sharp resonant bandwidth of the sensor provides a highly sensitive measurement, while optimizing the binding process of a respective antigen to the sensor surface is important for maximizing the usefulness of the sensor. The two methods of functionalization used in this paper provide benefits over

previous versions, from improved sensitivity and detection limits, to decreased time required to prepare sensors for analyte detection. Continuing on the progress seen from the results in this paper, further work will focus on signal amplification strategies to push the limits and applications of the PC-TIR sensor for clinical sample testing.

Acknowledgements

This research was supported by the National Institute of Health (R01GM126571). Its contents are solely the responsibility of the authors, and do not necessarily represent the official views of the NIH.

References

- [1]. Heron M, "Deaths: Leading causes for 2017", National Vital Statistics Reports; 68(6) (2017).
- [2]. Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, Carson AP, et al., "Heart disease and stroke statistics—2019 update: a report from the American Heart Association", *Circulation*. 2019;139(10): e56–528 (2019). [PubMed: 30700139]
- [3]. Thygesen K, Joseph S Alpert JS, White HD et al., "Universal definition of myocardial infarction", *European Heart Journal*, Volume 28, Issue 20, Pages 2525–2538 (10 2007). [PubMed: 17951287]
- [4]. Fathil M, Arshad MM, Gopinath SC, Hashim U, Adzhri R, Ayub R, Tang T-H, et al., "Diagnostics on acute myocardial infarction: Cardiac troponin biomarkers", *Biosensors and Bioelectronics*, 70, 209–220 (2015). [PubMed: 25841117]
- [5]. Reichlin T, Hochholzer W, Bassetti S, Steuer S, Stelzig C, Hartwiger S, Mueller C, et al., "Early Diagnosis of Myocardial Infarction with Sensitive Cardiac Troponin Assays", *New England Journal of Medicine*, 361(9) (2009).
- [6]. Aguero Villarreal VE, "Oriented immobilization of antibodies through recombinant protein-G on assembled gold nanorods for label-free biosensing applications", Available from Dissertations & Theses @ University of Texas - San Antonio; ProQuest One Academic. (1906287126). (2017).
- [7]. Syahir A, Usui K, Tomizaki K-Y, Kajikawa K, Mihara H, "Label and Label-Free Detection Techniques for Protein Microarrays", *Microarrays*, 4, 228–244 (2015). [PubMed: 27600222]
- [8]. Dorraj GS, Rassae MJ, Latifi AM, Pishgoo B, & Tavallaei M "Selection of DNA aptamers against Human Cardiac Troponin I for colorimetric sensor based dot blot application", *Journal of Biotechnology*, 208 (2015).
- [9]. Sharma S, Jackson PG, Makan J, "Cardiac Troponins", *Journal of clinical pathology*, 57(10), 1025–1026 (2004). [PubMed: 15452153]
- [10]. Chryssis AN, Saini SS, Lee SM, Yi H, Bentley WE, Dagenais M, "Detecting hybridization of DNA by highly sensitive evanescent field etched core fiber Bragg grating sensors," in *IEEE Journal of Selected Topics in Quantum Electronics*, vol. 11, no. 4, pp. 864–872, Jul-Aug (2005).
- [11]. Chow E, Grot A, Mirkarimi LW, Sigalas M, Girolami G, "Ultracompact biochemical sensor built with two-dimensional photonic crystal microcavity", *Optics Letters*, 29, 1093–1095 (2004). [PubMed: 15181996]
- [12]. Chao CY, Fung W, Guo LJ, "Polymer microring resonators for biochemical sensing applications", *IEEE J. Sel. Top. Quantum Electronics*. 12, 134–142 (2006).
- [13]. Cunningham BT, Li P, Schulz S, Lin B, Baird C, Gerstenmaier J, Genick C, Wang F, Fine E, Laing L, "Label-free assays on the BIND system", *J. Biomol. Screen*. 9, 481–490 (2004). [PubMed: 15452334]
- [14]. Dallo SF, Zhang B, Denno J, Hong S, Tsai A, Haskins W, Ye JY, Weitao T, "Association of *Acinetobacter baumannii* EF-Tu with Cell Surface, Outer Membrane Vesicles, and Fibronectin", *Sci. World J* 2012, 128705 (2012).
- [15]. Fan XD, White IM, Shopoua SI, Zhu HY, Suter JD, Sun YZ, "Sensitive optical biosensors for unlabeled targets: a review", *Anal. Chim. Acta* 620, 8–26 (2008). [PubMed: 18558119]
- [16]. Ramachandran A, et al., "A universal biosensing platform based on optical micro-ring resonators", *Biosensors & bioelectronics*. 23 939–44 (2008). [PubMed: 17964774]

- [17]. Schneider BH, Edwards JG, Hartman NF, “Hartman interferometer: versatile integrated optic sensor for label-free, real-time quantification of nucleic acids, proteins, and pathogens”, *Clinical Chemistry*, Volume 43, Issue 9, 1 September 1997, Pages 1757–1763 (1997). [PubMed: 9299972]
- [18]. Ye JY, Guo Y, Norris TB, Baker JR, Novel photonic crystal sensor patent issued Patent no. 7,639,362, (2009).
- [19]. Ye JY, Ishikawa M, “Enhancing fluorescence detection with a photonic crystal structure in a total-internal-reflection configuration,” *Opt. Lett.* 33, 1729–1731 (2008). [PubMed: 18670518]
- [20]. Zhang B, Tamez-Vela J, Solis S, Bustamante B., Peterson R, Rahman S, Morales A, Tang L, Ye JY, “Detection of Myoglobin with open-cavity and label-free photonic crystal biosensor”, *Journal of Medical Engineering*. 2013:808056 (2013). [PubMed: 27006922]
- [21]. Zhang B, Dallo S, Peterson R, Hussain S, Tao WT, Ye JY, “Detection of anthrax lef with DNA-based photonic crystal sensors”, *Journal of Biomedical Optics*. 2011:16 (2011).
- [22]. Zhang B, Morales AW, Peterson R, Tang L, Ye JY, “Label-free Detection of Cardiac Troponin I with a Photonic Crystal Biosensor”, *Biosens. Bioelectron.* 2014 August 15; 58: 107–113, (2014). [PubMed: 24632136]
- [23]. Guo Y, Ye JY, Divin C, Thomas TP, Myc A, Bersano-Begey TF, Baker JJR, Norris TB, “Real-Time Biomolecular Binding Detection Using a Sensitive Photonic Crystal Biosensor”, *Anal Chem.* 2010; 82:5211–5218 (2010). [PubMed: 20486666]
- [24]. Guo YB, Divin C, Myc A, Terry FL, Baker JR, Norris TB, Ye JY, “Sensitive molecular binding assay using a photonic crystal structure in total internal reflection”, *Optics Express*. 2008; 16:11741–11749 (2008). [PubMed: 18679444]
- [25]. Dorraj GS, Rassae MJ, Latifi AM, Pishgoo B, Tavallaei M “Selection of DNA Aptamers against Human Cardiac Troponin I for Colorimetric Sensor Based Dot Blot Application.”, *J. Biotechnol.* 2015, 208: 80–86. [PubMed: 26003883]

Figure 1.
Binding sequence for immobilization of cTnI antibodies on the sensor surface

Figure 2. Schematic diagram of experimental setup. PC-TIR Sensor is connected to a microfluidic system and coupled into the probe laser pathway.

Figure 3.

(a) Image received by a CCD camera with pure water in channels. (b). Image of reflected laser with ethylene glycol & water mixtures in channels. (c) Relative shift of resonant wavelength for corresponding ethylene glycol solution using experimental setup with white light source. (d) Relative shift of dark line with microchannels containing different mixtures of ethylene glycol in water compared to water reference channel.

Figure 4.

Binding kinetics for each step of the functionalization process is described in individual plots. Stage I shows baseline during water washes. Stage II shows binding effects that occur during sample incubation. Stage III portrays the final respective shift for each solution following a water wash. (a) Immobilization of Silane-PEG-Biotin on sensor surface. (b) Binding of streptavidin to biotin molecules on PEG. (c) RPG binding with streptavidin molecules. (d) Immobilization of anti-cTnI on RPG for orientation. (e) SEA blocker adsorption onto surface to prevent non-specific binding.

Figure 5. Final shift of cTnI for each method of functionalization. Red bars show shifts when anti-cTnI aptamers are used as the recognition element. Blue bars show shifts when RPG is used to bind anti-cTnI.