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Functionalized silicon dioxide self-referenced plasmonic chip as point-of-care biosensor for stroke biomarkers NT-proBNP and S100β

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ABSTRACT

Surface plasmon resonance (SPR) biosensors are often used in the detection of solid, liquid or gaseous samples in diagnostics, pharmaceutics and military defense. Plasmon waveguide resonance (PWR) mode is obtained when a dielectric waveguide layer is added to the metal film. In this study, a self-referenced PWR (SRPWR) silicon dioxide (SiO_2) chip was examined. The self-referenced measurement is important to compensate for temperature fluctuations, other instabilities and allows RI signal measurement without an additional reference sample, thus minimising the sample volume needed. The chip was fabricated with a multi-layer of metals and dielectrics, consisting of a 420 nm SiO_2 layer, a 40 nm Ag layer and another 480 nm SiO_2 layer. This chip was shown to give one internal plasmon excited on the bottom interface SiO_2/Ag, which is used as self-reference in the detection. The top layer acts as a waveguide layer and can be designed to give modes with ultrahigh penetration depth. A direct assay was developed, where the recognition molecule (specific antibody) was immobilized onto the SiO_2 plasmonic chip surface, via a covalent coupling protocol based on 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde. The SRPWR biosensor was developed for the sensing of two chosen stroke biomarkers: NT-proBNP and S100β, which are sensitive and specific for stroke diagnostics. For both biomarkers, a linear decreasing pattern in the RI signal was recognized with the increasing biomarkers concentrations. Biomarkers detection was conducted in deionized water and validation was done in spiked porcine plasma. The SiO_2 based plasmonic chip demonstrates a limit-of-detection of less than 1 ng/mL that is clinically relevant for both stroke biomarkers.

1. Introduction

Surface plasmon resonance (SPR) biosensors are often used in the detection of solid, liquid or gaseous samples, since their origin in 1983 [1]. Sensing is conducted directly, without labeling, thus allowing real – time measurements. They require small sample sizes, and exhibit highly sensitive, quantitative and reproducible results. These advantages have lead to applications in diagnostics, pharmaceutics and military defense [2]. SPR is advantageous over other various optical biosensors, such as fluorescence, interferometry, spectroscopy and optical waveguides, as they often require the use of labels and multi-step detection protocols [3]. Moreover, SPR biosensors are characterized as zero background techniques because the analyte is the only signal source. Therefore, they enable highly sensitive measurements. In addition, transmitting conduits are not needed for the signal measurement in SPR biosensors, because optical signals travel in an open path [4]. SPR biosensors are categorized as angle, intensity, wavelength, polarization modulation or phase sensors. They are based on a quantum electromagnetic phenomenon that arises when a metal-dielectric interface interacts with light. At a certain angle of the light, the energy of the photons excites the free electrons (surface plasmons (SPs)) on the metal interface, which results in a dip in the reflectance [5]. The excited extended SPs

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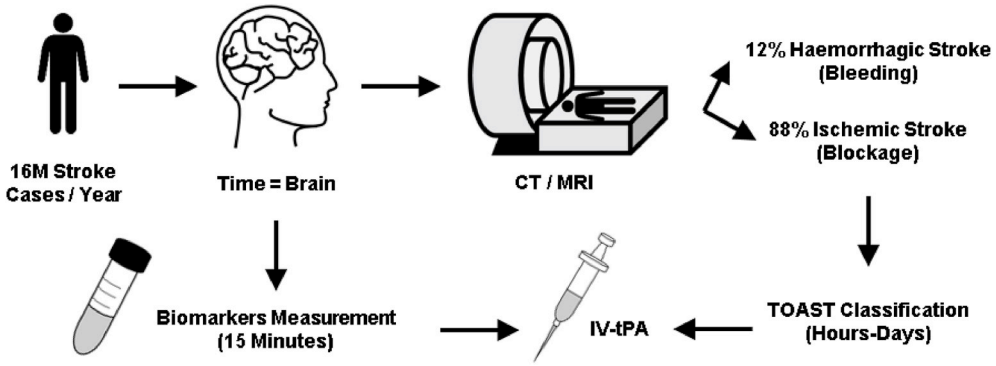

Fig. 1. Biomarkers Measurement for Stroke Prognosis. After stroke onset (16 million cases/year), brain cells die at a rapid rate and time-limited treatment (intravenous tissue plasminogen activator (IV-tPA)) must be administered immediately for greater effectiveness. A CT/MRI scan is performed in order to classify between ischemic (blockage) and haemorrhagic (bleeding) stroke. The Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification is then applied to underlay the stroke mechanism and allow for the administration of treatment. However, this process can take between hours to days. Integrating the use of n-terminal pro brain natriuretic peptide (NT-proBNP) and S100β biomarkers measurement (15 min) using a point-of-care biosensor can significantly shorten stroke prognosis time.

are considered as compressive electromagnetic surface waves on the metal interface. These surface waves are sensitive to refractive index (RI) shifts in the medium near the metal interface, which may quantitatively shift the resonant wavelength or the incident light angle [6]. In order to excite the SPs, the incident light must be TM – polarized and have enough momentum along the interface. This can be achieved by using refraction and diffraction elements such as prism, waveguide and grating coupling. The most efficient way is the commonly used setup of a prism based SPR biosensor, in which the metal surface is placed directly on top of the prism surface. The angle of incidence need to be higher than the critical angle for total internal reflection (TIR). The metal interface on the SPR chip that is used for the generation of plasmon waves is critical for the SPR biosensor performance [7].

Several metal interfaces were tested for their use in SPR biosensors, such as gold, silver, aluminium, copper, titanium, sodium, indium and chromium. Different metals exhibit different limitations in SPR biosensing, in terms of the metal atmospheric inertness, resonance sharpness, compatibility with the chemicals needed for the assay functionalization and cost. Gold is the most well studied and practically used metal in SPR biosensors, because of several important advantages that include having a strong SPR dip, its inertness and ease of functionalization for biomolecules immobilization [8]. Other metals showed significant limitations. For example, silver is sensitive to oxidation [9], copper and aluminum exhibit low sensitivities, sodium is violently reactive and indium is expensive [5]. A bimetallic layer of silver and gold has also been explored, which enables the combination of the advantages of both metals, while compensating on their disadvantages. The silver layer maintains the surface plasmon wave, while the gold layer ensures a chemically stable surface for the biomolecule immobilization [10]. In order to excite the SPs, an incorporation of a second material layer is also required, either above or below the metal interface. Both of the materials are selected carefully so as to allow for the resonance condition [5]. In recent years, silicon dioxide (SiO_2) chips have been increasingly used in various biosensor applications [11–13]. SiO_2 is a good SPR chip cover candidate because of its higher thermal stability and low cost [11]. The thermal stability of the chip surface is important for the immobilization of the bio-specific capture entity, which may involve high temperature conditions. Furthermore, the ease and cost effectiveness of SiO_2 processing makes it a good candidate as an SPR chip [12]. Studies have also shown that the sensitivity of bare gold chip has no significant difference as compared to the SiO_2 covered gold chip [13].

In this study, a novel self-referenced SiO_2 chip was examined. The chip was fabricated with a multi-layer of insulator-metal-insulator (IMI), consisting of a layer of 420 nm SiO_2, a layer of 40 nm silver and another top layer of 400 nm SiO_2. This chip was shown to give one plasmon excited at the internal SiO_2/Ag interface that is used as self-reference, while the other mode behaving like long range SPR with a high penetration depth controllable from few hundred nm till few microns [14]. This later mode is in fact a guided mode. The plasmon waveguide resonance (PWR) is a mode obtained when a dielectric waveguide layer is deposited on top of the metal film. Both transverse-electric (TE) and transverse-magnetic (TM) polarizations can be used to excite guided modes, which exhibiting narrow resonances, thus improved figure of merit. Isaacs et al. [15] had recently used this property in PWR sensor for determining the orientation of molecules on the surface. The addition of the bottom SiO_2 layer to the PWR sensor generates a self-referenced PWR (SRPWR), benefiting both from the higher figure of merit and the accurate measurement due to the self reference. The self referenced measurement is important to compensate for temperature fluctuations and other instabilities. This also allows to conduct accurate measurements of the RI signal without the need for an additional reference sample, thus minimising the sample volume needed.

A direct assay format was developed, where the recognition molecule was immobilized onto the SiO_2 plasmonic chip surface. Therefore, after introducing the sample, the target analyte is bonded to the SPR chip surface as well. This direct assay format is usually used for the detection of medium and large molecules with a molecular weight of 10 kDa [16]. Immobilizing the recognition molecule onto the SPR chip surface is an essential step, that is highly important and can affect the SPR biosensor performance. The immobilization can be conducted via different methods, including physical absorption, hydrophobic electrostatic interactions and covalent coupling (carboxylic, thiol or aldehyde groups) [2]. A covalent coupling immobilization protocol was utilized based on 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde, in order to covalently bind the recognition molecule onto the SiO_2 chip surface. Moreover, the SPRWR biosensor was developed in order to detect two particular stroke biomarkers, n-terminal pro brain natriuretic peptide (NT-proBNP) (8.5 kDa) and S100β (10 kDa), which are sensitive and specific for stroke diagnostic (Fig. 1). Stroke is among the world's top common causes of death, with 16 million stroke cases, and 7 million deaths annually [17,18]. The phrase ‘time is brain’ indicates that the effectiveness of stroke care is directly associated with timely diagnosis and administration of time limited treatment (intravenous tissue plasminogen activator (IV-tPA)) [19,20]. Current stroke diagnosis is mostly based on clinical examination, of CT/MRI scan and applying the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification. However, 35% of the patients still remain as classified with an undetermined stroke mechanism [21]. Recent studies have highlighted that stroke diagnosis can be improved by introducing blood-based biomarker measurements into the conventional scheme, in order to save critical time [22]. It was found that elevated plasma levels of NT-proBNP $\geq$ 0.5 ng/mL [23–37] and S100β $\geq$ 0.1 ng/mL [38–44] are predictive of stroke mechanisms. These low cut-off values make it a necessity to have a highly sensitive and rapid point-of-care biosensor capable of measuring these biomarkers below 1 ng/mL concentrations. Being miniature and cost effective, the SPR biosensor platform system is

suitable to become a point-of-care instrument when protocols for specific sensing are developed on its chips. This is the motivation of this work.

2. Materials and methods

2.1. Equipment

The SPR biosensor platform (SPR H5, PhotonicSys [45]) was used for the RI signal acquisition. The SRPWR SiO_2 plasmonic chip was used inside the SPR biosensor platform in order to allow for specific biomarkers detection. Ultrasonic cleaner (SONICA model MH, Soltec) was used for thorough cleaning of the new SPRWR SiO_2 chips. Peristaltic pump (Masterflex L/S, Cole-Parmer) was used for the washing of the SPRWR SiO_2 chip, while it is integrated into the SPR biosensor platform. Field Emission Scanning Electron Microscope (FE-SEM, Supra 55, Carl Zeiss) was used in order to characterize the integrity of the SPRWR SiO_2 chip surface. Weighing balance (Mettler Toledo) was used to scale the necessary samples and chemicals for the plasmonic chip calibration, functionalization and biomarkers measurement.

2.2. Materials

The SiO_2–Ag–SiO_2 chips, referred to as "SiO_2 chips, or IMI chips, or SRPWR chips)", were purchased from PhotonicSys. Phosphate buffer saline (PBS, pH 7.4) (SKU P4417), bovine serum albumin (BSA) (SKU 7030), absolute ethanol (SKU 02856), tween 20 (SKU P7949), glutaraldehyde, 25% in H2O (SKU G5882) and 3-aminopropyltriethoxysilane, 99% (APTES) (SKU 440140) were purchased from Sigma-Aldrich for the functionalization of the SPR SiO_2 chips. Anti-NT-proBNP 15F11 Antibody (CAT#orb79568) and NT-proBNP peptide (CAT#orb81959) were purchased from Biorbyt while Anti-S100β 8B10 Antibody (CAT#4S37) and S100β protein (CAT#8S9b) were purchased from HyTest.

2.3. SPR biosensor calibration

The SPR biosensor platform is factory-calibrated. However, it should be further calibrated to adjust to specific work setups, in order to accommodate any temperature and humidity changes. The calibration can be achieved by monitoring the pixel value of solutions with a well-known RI. In this study, the calibration was conducted by monitoring the pixel value of diluted glycerol (5% v/v–25% v/v) solutions in deionized water (Fig. 2). Subsequently, the known RI value of the solution was used in order to calibrate the pixel value accordingly. The SPR biosensor platform detects the location of the dark line in pixels through a camera connection. Then, the shift in the RI signal is monitored with a line detection algorithm. The algorithm first identifies the change in pixels, which is after translated into a shift in RI.

2.4. Functionalization of the silicon dioxide chip

Multiple steps were conducted for the functionalization of the SiO_2 chip surface. The functionalization was based on APTES and glutaraldehyde, as schematically presented in Fig. 3. The cleaning and activation of the SiO_2 chip surface has a significant effect on the subsequent surface functionalization steps. Firstly, the SiO_2 chip was cleaned by wash with de-ionized (DI) water in ultrasonic bath for 20 min at room temperature (RT). This was conducted in order to remove inorganic and organic surface contaminants. Subsequently, the SiO_2 chip was soaked in piranha solution (H_2SO_4:H_2O_2 = 7:3) for 1 min at RT. The piranha solution was used for the further removal of the remaining organic contaminants and for the improvement of the surface hydrophilicity, by hydroxylating the SiO_2 chip surface [46]. The SiO_2 chip surface is activated when it is hydroxylated, and it is then ready for the silanization by APTES. In the silanization step, the SiO_2 chip was soaked in 2% (v/v)

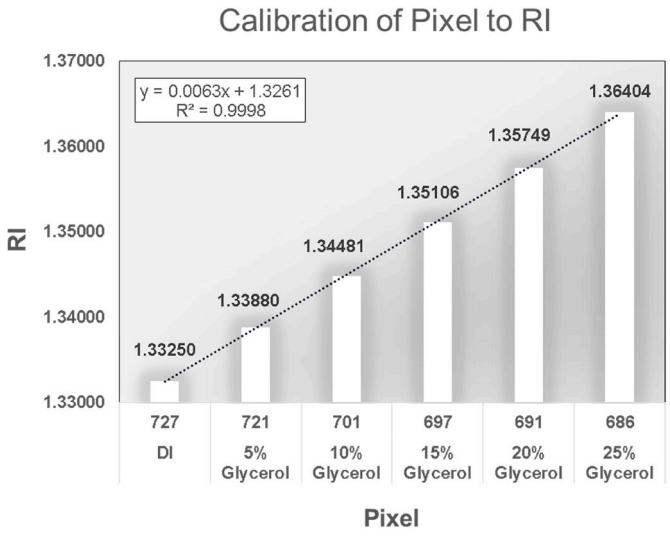

Fig. 2. Sensor Calibration of Pixel Value to Refractive Index. The surface plasmon resonance (SPR) biosensor platform detects the location of the dark line in pixels through a camera connection. The shift in the refractive index (RI) signal is then monitored with a line detection algorithm, which firstly identifies the change in pixels, before translating it into a shift in RI.

APTES solution in preheated ethanol with a drop of acetic acid for 60 min at RT. The silanization with APTES solution allows the formation of a monolayer silane (~2 nm) [46] and promote protein adhesion. Acetic acid was also added to the diluted APTES solution in order to orientate the amino groups outward from the interdigitated surface [47]. Later, after an additional 60 min incubation in the oven at 60 °C, the dried SiO_2 chip was soaked in 2.5% (v/v) glutaraldehyde in PBS solution for 60 min at RT. The chip incubation in the glutaraldehyde solution converts the amine-terminated surface into an aldehyde-terminated surface. After the generation of the aldehyde group, the chip was further incubated in a 20 μg/mL antibody solution (Anti-NT-proBNP or Anti-S100β) diluted in 0.05% (v/v) PBS-Tween20 for 18 h at 4 °C, in order to immobilize the specific antibody on the SiO_2 chip surface. Lastly, the SiO_2 chip was soaked in a 0.2% (w/v) BSA-PBS-0.05% (v/v) Tween20 solution, in order to block and maintain a constant pH on the surface, as well as to minimize non-specific binding and background signal [48].

2.5. Biomarker measurement

The specific biomarkers measurements were conducted using calibrated and functionalized SiO_2 plasmonic chips, that were placed inside the SPR biosensor platform. Fig. 4 presents the illustration of the SRPWR biosensor platform setup. As previously described in section 2.4, the SiO_2 plasmonic chip was firstly cleaned and later specifically functionalized. The functionalization was conducted in order to immobilize the bio-specific capture entity (antibody: either Anti-NT-proBNP or Anti-S100β) on the chip surface. The prism used in the SRPWR biosensor platform is made of SF11 glass. The TM polarized LD wavelength is 635 nm providing a diverging beam. Subsequently, the different samples containing increasing concentrations of the tested analyte (biomarkers: either NT-proBNP or S100β) were passed over the SiO_2 plasmonic chip for the detection of the specific biomarkers. The biomarkers were firstly diluted in deionized water for measurement and later in porcine plasma for validation. Lastly, the changes in RI values were recorded and analyzed. The self-reference feature of the SiO_2 plasmonic chip was used for all measurements. The biomarkers detection measurements were conducted both in self-reference mode and in reference-sample mode. This was done in order to validate that both modes of measurement provide the same RI signal. The self-referenced

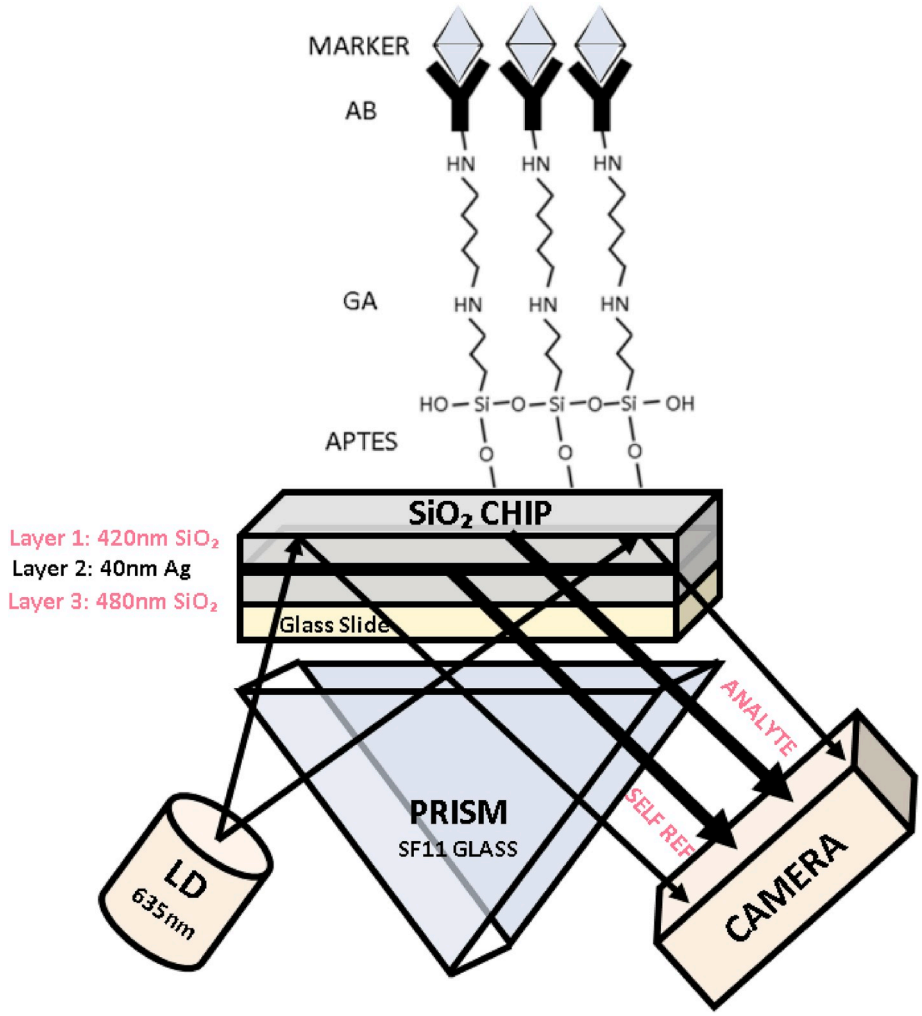

Fig. 3. Functionalization of the Silicon Dioxide Plasmonic Chip. The immobilization protocol, based on the utilization of 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde, was used for the covalent binding of a recognition molecule (specific antibody) onto the silicon dioxide surface. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

measurement is important to conduct accurate measurements of the RI signal without the need for an additional reference sample (Fig. 5). The top dark line corresponds to the self-reference SPR while the bottom dark line corresponds to the PWR mode. Before sample addition, the bottom dark lines correspond to air. After DI water sample addition, into both reference and analyte channels, two dark lines are seen. The

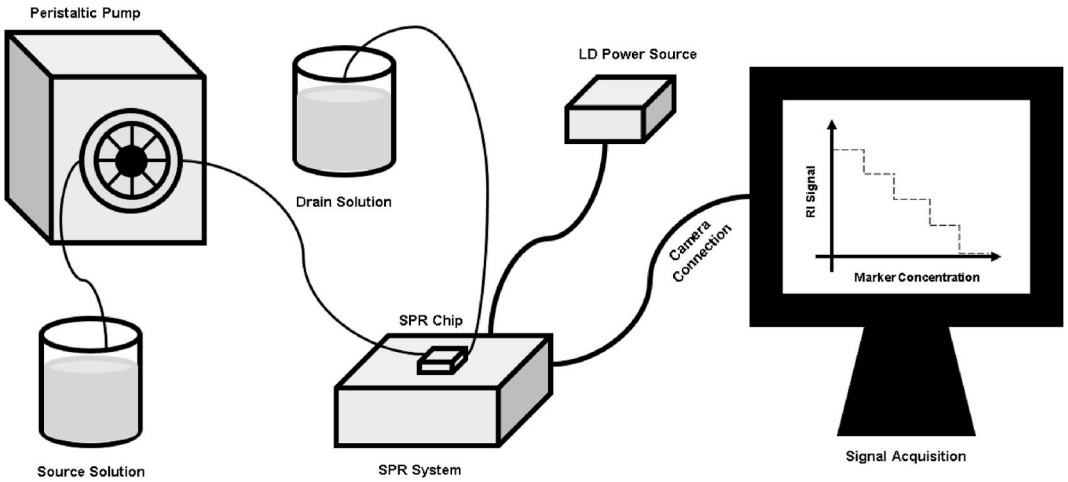

Fig. 4. Illustration of The Surface Plasmon Resonance Biosensor Platform Setup. The surface plasmon resonance (SPR) biosensor platform is connected to a laser diode (LD) power source. In addition, a camera was connected to the computer for signal acquisition through a customized software. The system is also connected to a peristaltic pump, via a first tube to the source solution and a second tube to the drain solution. For an image of the SPR biosensor platform system and connections to a pump, the reader is forwarded to our earlier paper [49].

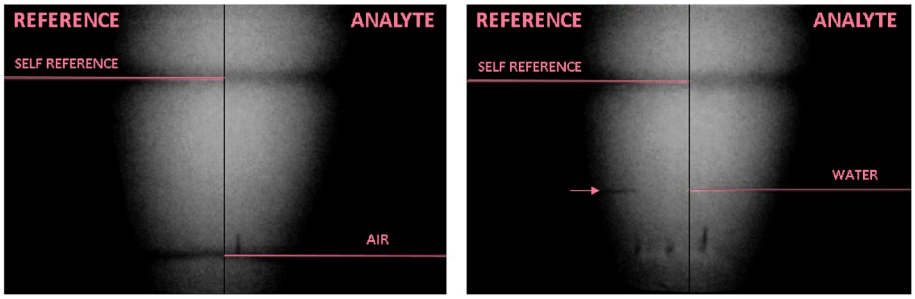

Fig. 5. Self-Referenced Silicon Dioxide Plasmonic Chip. The self-referenced plasmon waveguide resonance (SPRWR) SiO_2 chip allows for a stable self-reference measurement due to its multi-layered structure of: 420 nm SiO_2 - 40 nm Ag - 480 nm SiO_2, thus minimising the sample volume required. The top dark line is the self-reference line while the bottom dark line corresponds to the PWR mode. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

software red color marker covers the thin dark line, which is a proof of how thin it is, while it is clearly seen in the reference channel. The thinner dark line means much narrower resonance and therefore higher figure of merit. Moreover, the sensor has a wide dynamic range, as it can detect gases (see dark line corresponding to air) and liquids using the same chip. The IMI chip is designed to be compatible with the miniature SPR biosensor system platform [45] and the software that enables the monitoring of both the reference line as well as the two sample channels in parallel to the option of using a gold based chip [49].

2.6. Data analysis

The data was recorded and analyzed using SPSS 23.0 (IBM SPSS Statistics, version 23). Firstly, descriptive analysis was conducted on the pixel and RI values, in order to identify the signals differences. Moreover, in order to accurately report the signals obtained, values are reported as median and interquartile range (IQR). In addition, limit-of-detection was calculated for each biomarker measurement (NT-proBNP and S100β) in each sample medium tested (deionized water and porcine plasma). The calculation was based on the following equation:

$$Limit - Of - Detection\ (LOD) = \frac{(k \times SD)}{m}$$

The parameters were as follow: confidence level ($k = 3$); average standard deviation (SD refractive index units (RIU)) and calibration sensitivity m (RIU/(ng/mL)), the slope of the linear plot, for each biomarker measurement in each sample medium.

3. Results and discussion

3.1. Functionalization

As previously discussed, SiO_2 chips have been increasingly used in various biosensor applications [11–13]. SiO_2 is a good candidate as a SPR chip because of its higher thermal stability [11], the ease and cost effectiveness of SiO_2 processing [12]. Studies have also shown that the sensitivity of bare gold chips has no significant difference as compared to the SiO_2 covered gold chip [13]. Moreover, higher sensitivity can be obtained in angular mode when a thin dielectric layer with high RI is deposited (below the cutoff for guided mode excitation), while the figure of merit remains the same [50,51]. On the other hand, the sensitivity and the figure of merit can be both enhanced in the spectral mode [52]. The chip examined in this study is novel because it is constructed with thick dielectric layers below and above the metal film, in order to excite both the self reference plasmon and the higher figure of merit guided mode. The chip was fabricated with a multi-layer of insulator-metal-insulator, consisting of a 420 nm SiO_2 layer, a 40 nm silver layer then another layer of 480 nm SiO_2. A covalent coupling immobilization protocol was examined in order to immobilize the recognition molecule on the SPR chip surface and to develop a direct assay format. The protocol is based on the utilization ofAPTES and glutaraldehyde, in order to covalently bind the recognition molecule onto the SiO_2 chip surface. Previous studies, had examined the covalent coupling immobilization protocol on SiO_2 surface, based on APTES and glutaraldehyde [46–48]. However, it was never tested for use in SPR sensing. The functionalization of the SiO_2 plasmonic chip required the use of an ultrasonic bath to remove any inorganic and organic contaminants. Then, the chips were cleaned and activated with piranha solution to improve the surface hydrophilicity via the hydroxylating of the silicon surface. Silanization with APTES was then conducted for the formation of a monolayer silane (~2 nm) [46] and the promotion of protein adhesion. The chips were subsequently soaked in gluteraldhyde solution in order to convert the amine-terminated surface into an aldehyde-terminated surface, before the antibody coupling onto the substrate surface and blocking with BSA. The surface modifications of the SiO_2 plasmonic chip after each functionalization step were validated by monitoring the changes in the pixel value of deionized water (Fig. 6). Adsorptions on the SiO_2 surface changes the SPR resonance angle θ_{SPR}, and consequently the RI of the analyte solution near the SiO_2 layer, which results in a change in the pixel value measured by the SPR biosensor. As shown in Fig. 6, the pixel value decreased over the functionalization steps. The decrease in pixel value indicated a shift in the SPR dip upwards, meaning the SPR resonance angle θ_{SPR} increased as expected. A significant change is visible following the two functionalization steps of antibody (pixel 6 A: 722.3 and 6 B: 721.0) and BSA protein (pixel 6 A: 718.8 and 6 B: 716.9) immobilizations. Moreover, after introducing increasing levels of the biomarkers (NT-proBNP and S100β) concentrations, an increase in pixel values are shown. For NT-proBNP, a steeper increase is visible (Fig. 6A), with the pixel value increasing from 718.3 to 724.1 in the highest concentration tested of 10 ng/mL. For S100β, a milder pattern is visible (Fig. 6B), with the pixel value increasing from 714.7 to 718.9 in the highest concentration tested of 10 ng/mL. To conclude, the changes on the SiO_2 plasmonic chip surface were confirmed by the observation of a negative shift in the pixel values, which is equivalent to a positive shift in the SPR dip.

3.2. NT-proBNP measurement

N-terminal pro brain natriuretic peptide (NT-proBNP) was chosen as a stroke biomarker candidate as it is both well-studied and has also shown excellent diagnostic accuracies. NT-proBNP, 76-amino acid peptide, is brain natriuretic peptide (BNP) biologically inactive derivative [53,54]. The neurohormone BNP is secreted after excessive stretching of heart muscle cells [55] from the heart ventricles [56]. Most assays prioritize the detection of NT-proBNP over BNP because of its longer half-life and higher molecular weight (NT-proBNP - Mw: 8.5 kDa; half-life: 120 min vs. BNP - Mw: 3.5 kDa; half-life: 20 min) [57]. NT-proBNP elevated plasma levels ≥0.5 ng/mL were found to be predictive of stroke mechanisms [23–37]. The low cut-off value of less

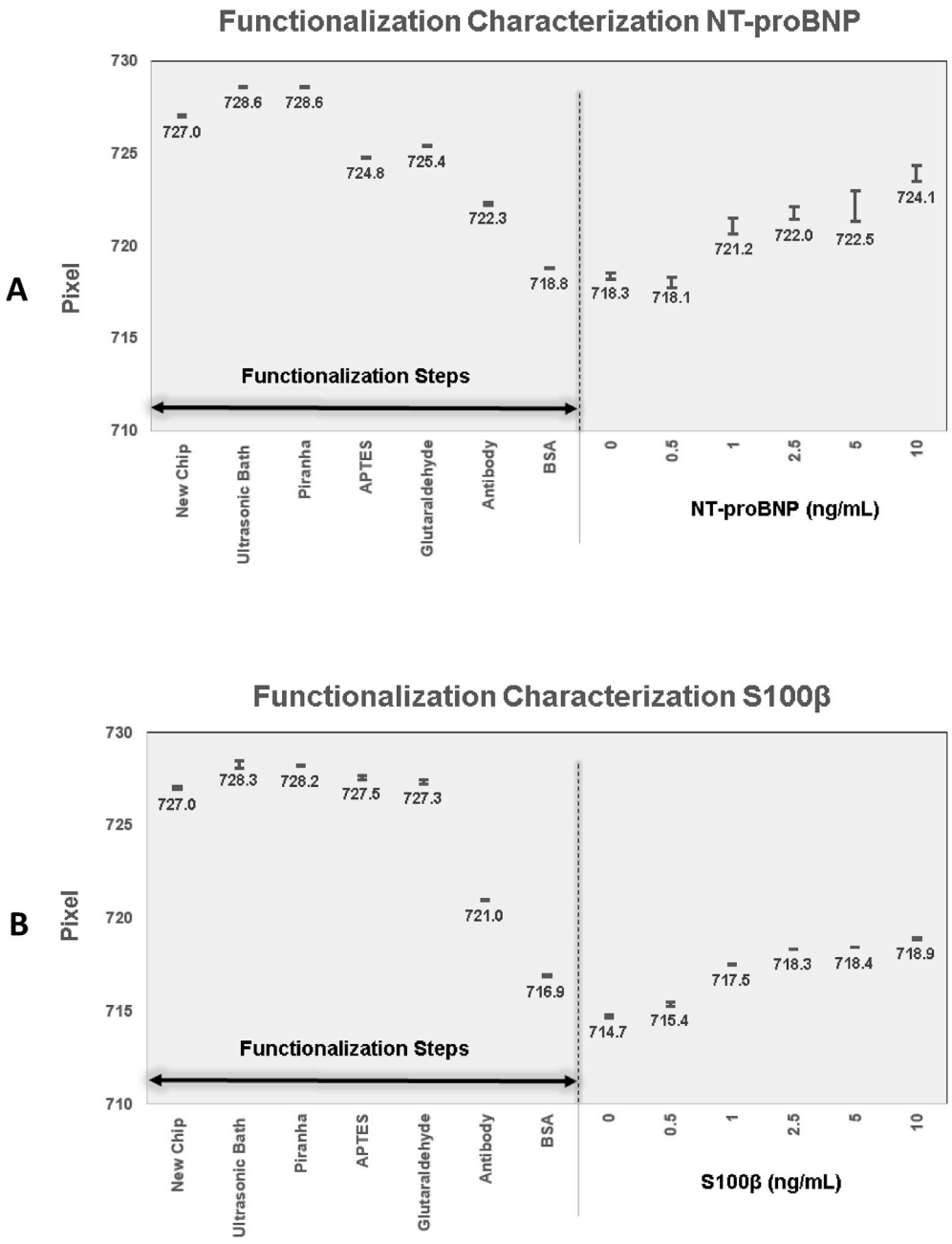

Fig. 6. Functionalization Characterization of the Silicon Dioxide Chip Surface. Monitoring the pixel value after each of the functionalization steps, and detection of several concentrations of: A) NT-proBNP; B) S100β. The signal is the pixel values, which are representing the resonance angle.

then 1 ng/mL set a need for a sensitive analytical device. Combined with the additional requirements for stroke diagnostics, there is a need for a highly sensitive and rapid point-of-care biosensor that can measure NT-proBNP in sub-nano concentrations. Therefore, a sensitive biosensor based on SPR detection was developed. The SiO_2 plasmonic chip was functionalized with specific anti-NT-proBNP antibody, based on an APTES and glutaraldehyde reaction. Subsequently, the different samples containing increasing NT-proBNP concentrations between 0.5 ng/mL to 10 ng/mL diluted in deionized water, were introduced onto the plasmonic chips. NT-proBNP detection showed an inverse, linear relationship between the RI signal and NT-proBNP concentrations. This decreasing pattern in the RI signal with increasing NT-proBNP concentrations in deionized water was quantified by the following linear equation: $Y = - 0.0008X + 1.3337$ ($R^2 = 0.9542$) (Fig. 7A). In addition, the LOD in deionized water was calculated and identified as 0.1275 ng/mL NT-proBNP. The calculation (assuming $k = 3$ confidence level) was based on the following two specific parameters: sensitivity (0.0008 (RIU/(ng/mL))) and the average SD (0.000034 (RIU)). The sensing of NT-proBNP with SPR technology was previously examined by Binbin Luo et al. [58], with excessively tilted fiber gratings (Ex-TFGs) (LOD of 0.5 ng/mL), and Harpaz et al. [49] with a gold-silver plasmonic chip (LOD of 0.12 ng/mL). In addition, the sensing of BNP with SPR technology was previously examined only with gold as the metal interface in the plasmonic chip. Yuji Teramura

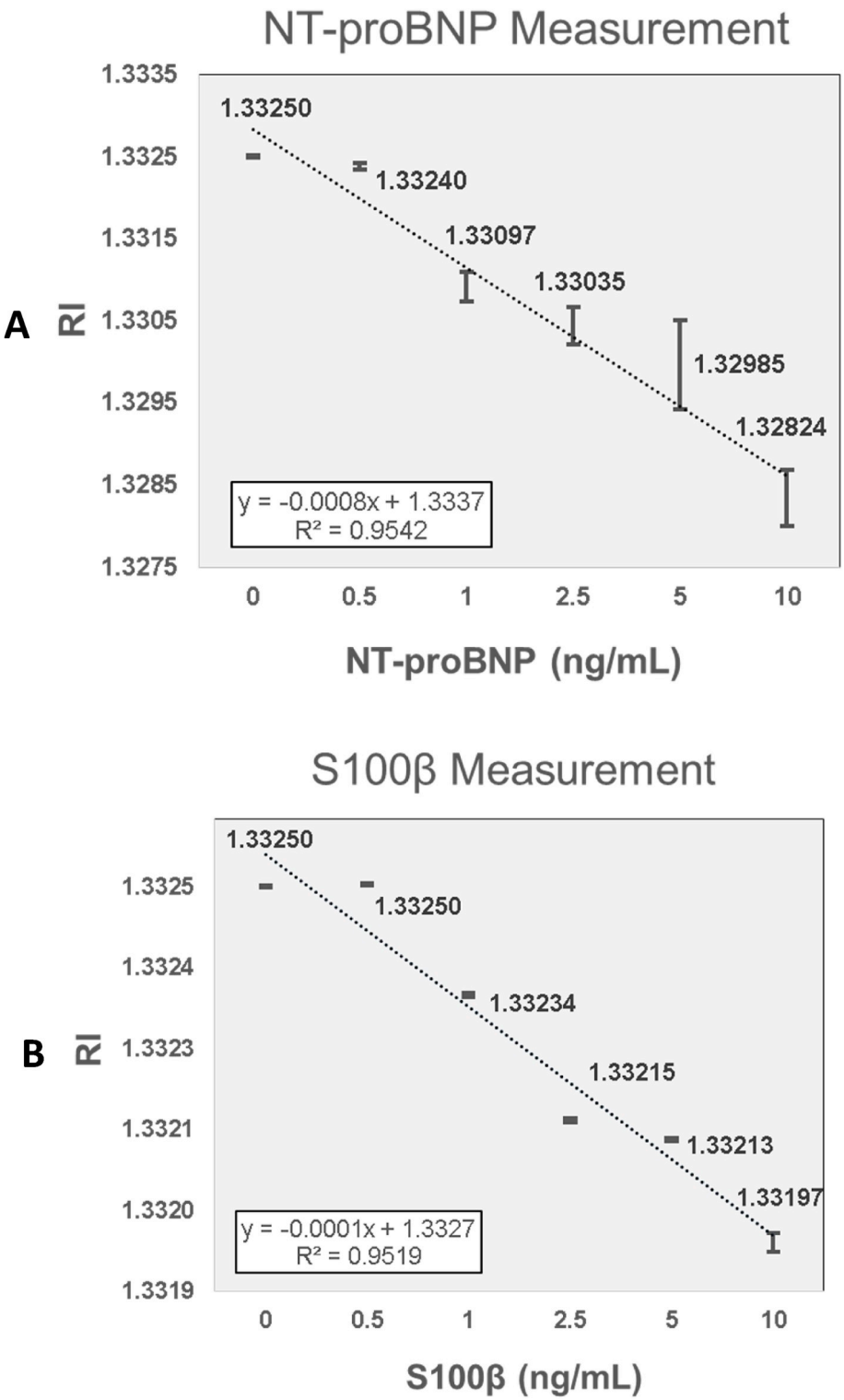

Fig. 7. Biomarkers Measurement with Silicon Dioxide Plasmonic Chip. Monitoring the refractive index (RI) value of various clinically relevant concentrations of the stroke biomarkers: A) NT-proBNP and B) S100β, diluted in deionized water.

et al. [59] explored BNP detection with a sandwich biosensor layout, Jang et al. [60] also examined a sandwich biosensor layout while utilizing aptamer as the bio-specific capture entity, and Kurita et al. [61] developed BNP detection using a portable SPR sensor system combined with a microfluidic device. Our results demonstrate a novel, sensitive and self-referenced SiO_2 plasmonic chip utilized as a SPR biosensor for NT-proBNP sensing in clinically relevant limit-of-detection for stroke diagnosis. The system used is also miniature and cost effective,

therefore it can be used for point-of-care diagnostics.

3.3. S100β measurement

S100β was also chosen as a stroke biomarker candidate as it has demonstrated great diagnostic accuracies. S100β is part of the non-ubiquitous Ca^{2+}-modulated protein family. It is an intracellular 10 kDa glial protein, secreted mainly from astrocytes [62]. This biomarker has a significant contribution to the differentiation, growth and repair of nerves [63–65]. It is mainly found in high levels in the cerebral astroglial compartment, peripheral Schwann cells, extraneuronally in melanocytes, adipocytes and chondrocytes [66]. Previous studies found that S100β elevated plasma levels ≥0.1 ng/mL are predictive of stroke mechanisms [38–44]. Again, this low cut-off value of less than 1 ng/mL makes it necessary to develop a highly sensitive and rapid point-of-care biosensor that can measure S100β below 1 ng/mL concentrations. Similarly, the SiO_2 plasmonic chip was functionalized with a specific anti-S100β antibody based on an APTES and glutaraldehyde reaction. Subsequently, the different samples containing increasing S100β concentrations between 0.5 ng/mL to 10 ng/mL diluted in deionized water, were introduced onto the plasmonic chips. S100β detection showed a similar inverse, linear relationship between the increasing S100β concentrations ad the decreasing RI signal. This decreasing pattern in the RI signal with increasing S100β concentrations in deionized water was quantified by the following linear equation: Y = - 0.0001X + 1.3327 (R^2 = 0.9519) (Fig. 7B). In addition, the LOD in deionized water was calculated and identified as 0.18 ng/mL S100β. The calculation (assuming k = 3 confidence level) was based on the following two specific parameters: sensitivity (0.0001 (RIU/(ng/mL))) and the average SD (0.000006 (RIU)). S100β sensing based on SPR technology was previously examined only by Harpaz et al. [49] with a gold-silver plasmonic chip (LOD of 0.75 ng/mL). Previous studies only examined the use of SPR technology for the testing of protein – protein interactions of S100β, by Seitz et al. [67] and Leclerc [68]. Our results demonstrate a novel, sensitive and self-referenced SiO_2 plasmonic chip utilized as a SPR biosensor for S100β sensing in clinically relevant limit-of-detection for stroke diagnosis.

3.4. Validation in plasma

The sensing of the stroke biomarkers, NT-proBNP and S100β, with the SiO_2 plasmonic chip, was later also validated in plasma samples. The biomarkers were spiked into porcine plasma, which was used as a substitute to human plasma, because it is the sample type that is mostly used for the diagnostic testing of NT-proBNP and S100β. In addition, 90 nm SiO_2 layer was added onto the top of the plasmonic chip, in order to optimize the sensing range for the RI range of the plasma samples. The detection of increasing NT-proBNP concentrations in the spiked plasma samples showed consistent results with an inverse linear pattern of decreasing RI signal. This decreasing pattern in the RI signal with increasing NT-proBNP concentrations in plasma was quantified by the following linear equation: Y = - 0.00003X + 1.3624 (R^2 = 0.9802) (Fig. 8A). In addition, the LOD in plasma was calculated and identified as 1.3 ng/mL NT-proBNP. The calculation (assuming k = 3 confidence level) was based on the following two specific parameters: sensitivity (0.00003 (RIU/(ng/mL))) and the average SD (0.000013 (RIU)). Moreover, the detection of increasing S100β concentrations in the spiked plasma samples also showed consistent results with an inverse linear pattern of decreasing RI signal. This decreasing pattern in the RI signal with increasing S100β concentrations in plasma was quantified by the following linear equation: Y = - 0.0005X + 1.3631 (R^2 = 0.9168) (Fig. 8B). In addition, the LOD in plasma was calculated and identified as 0.096 ng/mL S100β. The calculation (assuming k = 3 confidence level) was based on the following two specific parameters: sensitivity (0.0005 (RIU/(ng/mL))) and the average SD (0.000016 (RIU)).

4. Conclusions

In this study, a functionalized, novel SiO_2 plasmonic chip was developed for the sensing of two specific stroke biomarkers, and utilized for use in an SPR point-of-care biosensor. This is the first known study whereby SiO_2 plasmonic chip was used in an SPR-based point-of-care device to detect blood-based biomarkers for stroke. Both NT-proBNP and S100β were chosen as the two stroke biomarkers, because extensive research has been conducted on them, with great diagnostic accuracy demonstrated. The novel SiO_2 plasmonic chip was shown to give one internal plasmon which is used as self-reference, while the other mode behaving like long range SPR has a high penetration depth controllable from few hundred nm till few microns [14]. In recent years, SiO_2 chips have been increasingly used in various biosensor applications [11–13] due to a higher thermal stability and low cost of SiO_2 material [11]. Thermal stability of the surface is a critical factor for the immobilization of bio - specific capture entity which may involve high temperature condition. Furthermore, the ease and cost effectiveness of SiO_2 processing makes it a good candidate as a SPR chip [12]. It is well understood that silver, gold and SiO_2 are suitable materials for the fabrication of SPR chips. Studies have also shown that the sensitivity of bare gold chip has no significant difference as compared to the SiO_2 covered gold chip [13]. The SPR biosensor is capable of detecting the difference in the biomarkers concentrations, by using a specifically functionalized plasmonic chip, immobilized with a bio-specific capture entity (antibody) that serve as a recognition element [69]. The specific binding of the analyte is detected as a shift in RI. The SiO_2 plasmonic chips were functionalized with specific antibodies, based on an APTES and glutaraldehyde reaction. Subsequently, the different samples containing increasing biomarkers concentrations between 0.5 ng/mL to 10 ng/mL, were introduced to the plasmonic chips. Pixel change was first detected by a camera, before being translated into a shift in RI using a customized software. NT-proBNP detection showed an inverse, linear relationship between the RI signal and NT-proBNP concentrations. This decreasing pattern in the RI signal with increasing NT-proBNP concentrations was quantified by the following linear equations, in deionized water: Y = - 0.0008X + 1.3337 (R^2 = 0.9542) (Fig. 7A), and in spiked plasma: Y = - 0.00003X + 1.3624 (R^2 = 0.9802) (Fig. 8A). In addition, the LOD was calculated and identified, in deionized water: 0.1275 ng/mL and in spiked plasma: 1.3 ng/mL NT-proBNP. Moreover, S100β detection showed a similar inverse, linear relationship between the increasing S100β concentrations ad the decreasing RI signal. This decreasing pattern in the RI signal with increasing S100β concentrations was quantified by the following linear equations, in deionized water: Y = - 0.0001X + 1.3327 (R^2 = 0.9519) (Fig. 7B), and in spiked plasma: Y = - 0.0005X + 1.3631 (R^2 = 0.9168) (Fig. 8B). In addition, the LOD was calculated and identified, in deionized water: 0.18 ng/mL and in spiked plasma: 0.096 ng/mL S100β. To conclude, the SRPWR biosensor was developed with the purpose of detecting two chosen stroke biomarkers NT-proBNP and S100β, which are sensitive and specific for stroke diagnostics. For both biomarkers, a linear decreasing pattern in the RI signal was recognized with the increasing biomarkers concentrations. The SRPWR SiO_2 plasmonic chip demonstrates a limit-of-detection of less than 1 ng/mL that is clinically relevant for both NT-proBNP and S100β as stroke biomarkers.

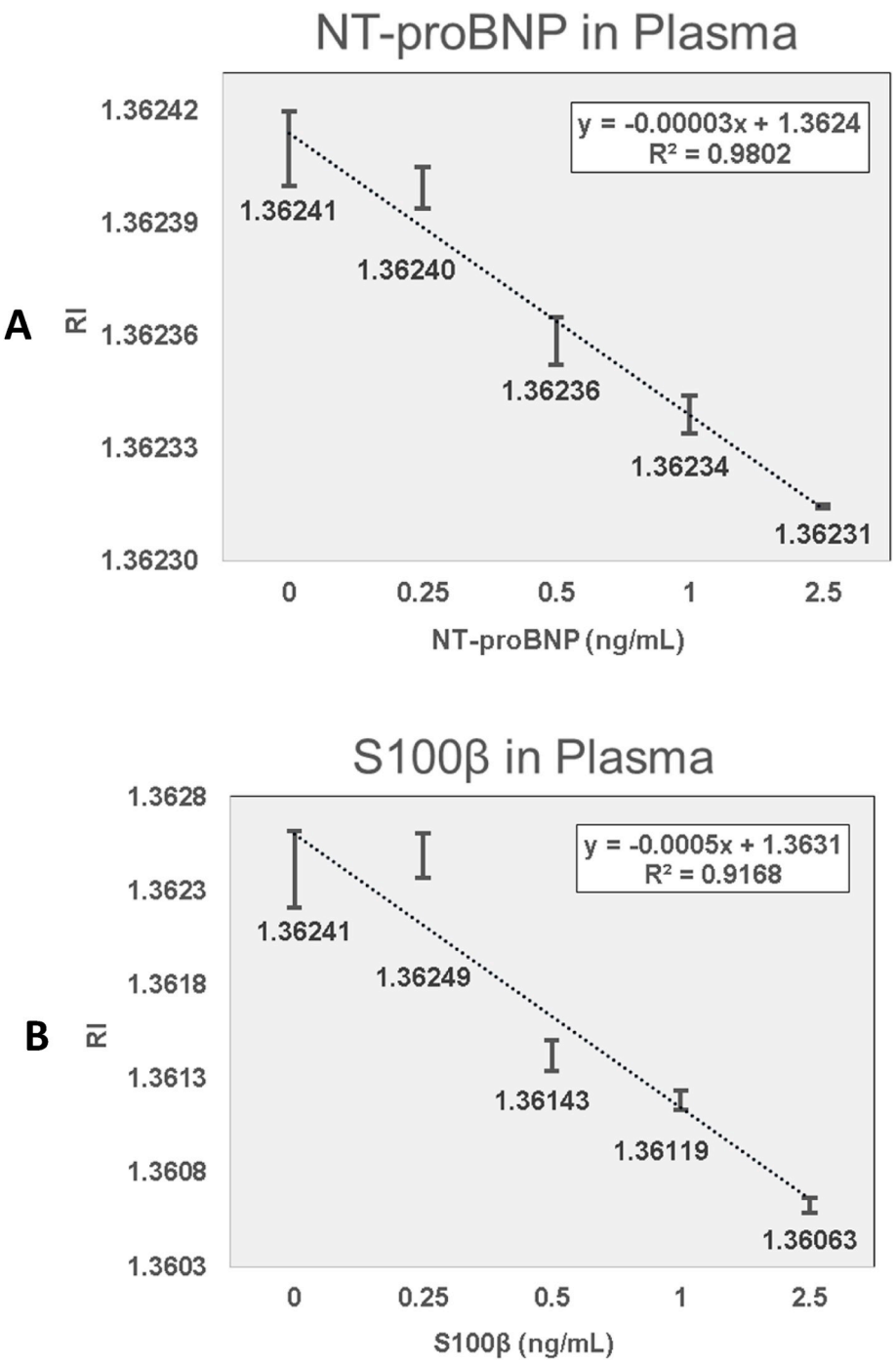

Fig. 8. Biomarkers Sensing Validation in Plasma with Silicon Dioxide Plasmonic Chip. Monitoring the refractive index (RI) value of various clinically relevant concentrations of the stroke biomarkers: A) NT-proBNP and B) S100β, in spiked porcine plasma samples.

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CRediT authorship contribution statement

Dorin Harpaz: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Brescia Koh:** Investigation. **Raymond C.S. Seet:** Writing - review & editing. **Ibrahim Abdulhalim:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Alfred I.Y. Tok:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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