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Abstract. The detection of thrombin based on aptamer binding is studied using two different optical fiber-based configurations: long period gratings coated with a thin layer of titanium dioxide and surface plasmon resonance devices in optical fibers coated with a multilayer of gold and titanium dioxide. These structures are functionalized and the performance to detect thrombin in the range 10 to 100 nM is compared in transmission mode. The sensitivity to the surrounding refractive index (RI) of the plasmonic device is higher than 3100 nm RIU in the RI range 1.335 to 1.355, a factor of 20 greater than the sensitivity of the coated grating. The detection of 10 nM of thrombin was accomplished with a wavelength shift of 3.5 nm and a resolution of 0.54 nM. ©2016 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.21.8.087005]

Keywords: surface plasmon resonance; long period fiber gratings; titanium dioxide; fiber optic refractometer; thrombin-binding aptamer.

Paper 160046RR received Jan. 23, 2016; accepted for publication Aug. 1, 2016; published online Aug. 18, 2016.

1 Introduction

Low cost analysis systems capable of providing measurements of analyte in a short-time period or even in real time at low concentration are paramount in many fields, such as medicine, drugs detection, or food safety. Fiber optic devices applied to biosensing are an appealing solution that can fulfill these requirements. It is important to recognize the advantages of these biosensors including their compatibility to a wide range of surface modifications, chemical-inertness, their compactness and cost effectiveness, and their potential for remote sensing.1

Thrombin is a key protein playing an important role in the blood coagulation cascade and in homeostasis.2,3 The concentration of thrombin in blood is quite variable and several clotting factors are affected by thrombin concentrations.4 The use of aptamer-based assays for detection and quantification of thrombin has several advantages due to their high specificity and affinity to its targets, considering their ability to fold into numerous tertiary conformations.5,6 Moreover, aptamers can be generated and synthesized in a large quantity in vitro in a very reproducible way.7,8 They are thermally and chemically very stable, increasing their use in time without losing its ability to bind specifically to their target.9,10

The detection of thrombin could be achieved through the manipulation of a specific thrombin-binding aptamer (TBA) that inhibits the activity of thrombin.11 This capability of regulating thrombin activity using synthetic compounds is a major mark in prevention of thrombosis.

The immobilization of the DNA on a waveguide surface leads to changes in the spectral response, which can be measurable with specific optical fiber components, such as long period fiber grating (LPFG) or surface plasmon resonance (SPR) structures. Typical LPFG and SPR fiber-based sensors show great potential to be used as biosensing devices coupled with the well-known advantages of optical fiber sensors.12–14 LPFG relies on coupling light from the core mode of a single-mode fiber (SMF) into cladding modes through the introduction of a periodic modulation in the refractive index (RI) of the fiber core.15 The period of the RI modulation is such that a phase matching condition between the core mode and a forward propagating cladding mode of an optical fiber occurs at a given wavelength:16

\[
\lambda = \left| n_{\text{eff}}(\lambda) - n_{\text{clad}}^{i}(\lambda) \right| \Lambda,
\]

where \(n_{\text{eff}}(\lambda)\) is the effective RI of the core mode at wavelength \(\lambda\), \(n_{\text{clad}}^{i}(\lambda)\) is the effective RI of the \(i\)th cladding mode, and \(\Lambda\) is the period of the LPFG. Several attenuation bands are formed in the transmission spectrum of the fiber.17

An evanescent field reaches outside the cladding perimeter, thus making LPFGs sensitive to the optical properties of materials surrounding the fiber. This characteristic has led to studies of LPFGs coated with a number of materials which are chosen to change their optical properties in response to an external stimulation.18

Optical fiber sensors based on SPR, on the other hand, have been studied using different configurations and applied to different geometries.19 SPR can be defined as a charge-density oscillation that, in certain conditions, can be generated at the interface of two media, a metal and a dielectric, with dielectric constants of opposite signs. The charge density oscillation is associated with an electromagnetic wave with the evanescent...
field at the interface decaying exponentially into both media.\textsuperscript{20}
The result is an attenuation band in the transmission spectra whose wavelength position is highly dependent on the external RI.

In this work, two different types of refractometric platforms using optical fibers devices, LPFGs and SPRs, were validated to be used as biosensing devices, for the detection of thrombin using a 15-mer single-stranded DNA aptamer-based probe as a model system for protein detection.

2 Materials and Methods

2.1 Reagents and Solutions

The thrombin oligonucleotide sequence (5'-amine-GGTTGCTGTTGG-3') with a synthesis scale of 0.2 μM, purification HPLC, and 5’ amine modification was purchased from Sigma-Aldrich (Spain). Human-α-thrombin was obtained from Haemalogic Technologies in a concentration of 1 mg mL\textsuperscript{-1} (50 mM sodium phosphate, 150 mM sodium chloride, pH 6.5, 3757 U mg\textsuperscript{-1}). The solutions of thrombin used in each assay were prepared by dilution in 10 mM of phosphate-buffered saline from Sigma-Aldrich (Spain) in Eppendorf's blocked with 1% bovine serum albumin, from Merck, aliquoted and stored at −80°C. Salts for buffer solutions were prepared in ultra-pure water, <15 MΩ cm at 25°C. Hydrochloric acid (HCl), isopropanol (IPA), sodium hypochlorite, ethanol, and magnesium chloride were purchased from Panreac (Spain), Tris(hydroxymethyl)aminomethane (Tris), 0.1 % (w/v) poly-L-lysine (PLL), potassium chloride (KCl), sodium chloride (NaCl), SSPE buffer 20x concentrate [1x = 0.150 M sodium chloride, 0.010 M sodium phosphate, 0.001 M ethylenediaminetetraacetic acid (EDTA)] and Tris-EDTA (TE) were obtained from Sigma (Germany). The buffers used in this work were aptamer resuspension buffer: TE (10 mM Tris pH 7.5 to 8.0; 1 mM EDTA); aptamer immobilization buffer: SSPE (1:10), (diluted from 20X SSPE); affinity buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM MgCl\textsubscript{2}) and measurement buffer (10 mM Tris-HCl pH 7.4, 100 mM KCl). All the reagents were of analytical grade.

2.2 Optical Fiber Sensors Fabrication

Both sensing structures were produced from the regular SMF28 from Corning, Inc., following a specific protocol. The LPFG sensors were produced by the electric-arc technique, as described by Rego et al.\textsuperscript{21} which consists of applying point-by-point short time discharges to the uncoated fiber. The period of the gratings was 396 μm, a value chosen to produce a resonance wavelength at 1550 nm matching the antisymmetric sixth order cladding mode (LP\textsubscript{16}) according to Ivanov and Rego.\textsuperscript{22} The grating attenuation band value was reached with a sensor length of 45 ± 5 mm.

The SPR sensing device was produced using the procedure previously described.\textsuperscript{25} Briefly, the plastic protective jacket was removed and a 10 mm long section of the fiber was chemically etched by immersion in an aqueous solution with 10 mL of 48% HF during 48 min at 22°C and 60% of humidity until the etched region became ~16 μm in diameter. The sensing region is located at the thinner section, where the evanescent field is able to excite the plasmonic wave. First, the etched region was coated with 2 nm of pure chromium (Cr) to improve the adhesion of gold (Au). Then a 16-nm thick film of Au was deposited around the fiber, followed by a 100-nm thick dielectric layer of TiO\textsubscript{2}. The thickness of both metal and dielectric layers was calculated in a previous publication\textsuperscript{25} to obtain a resonance in the C-band by using the transfer matrix formalism approximation for a SLAB multilayer system.

The metal/dielectric coatings were produced around the cylindrical fiber shape by thermal evaporation of pure titanium (Ti) in a controlled oxygen atmosphere using an electron beam evaporator (Auto 306, Edwards Ltd., United Kingdom) fitted with a rotary system, which improves the homogeneity of the coating.

2.3 Biosensing Principle

Both sensing surfaces were functionalized with PLL, followed by the immobilization of the DNA probe—TBA, for the specific recognition of thrombin. It is well-studied, 15-mer single-stranded DNA that shows good affinity and specificity against thrombin as has been described by Bock et al.\textsuperscript{26} and does not interact with other plasma proteins or thrombin analogues, such as gamma-thrombin.\textsuperscript{27}

The detailed biosensor principle is illustrated in Fig. 1. First, in Fig. 1(a), the sensing surface was cleaned with IPA and then rinsed with ultra-pure water and dried with nitrogen (N\textsubscript{2}). Then in Fig. 1(b), the surface was functionalized with PLL (60 min at room temperature). The polycationic nature of the PLL leads to its electrostatic attraction onto the fiber’s negatively charged surface, in an aqueous environment at neutral pH (7.4). After that in Fig. 1(c), the 5'-amine modified TBA (500 nM) was incubated...
for 60 min at room temperature for its covalent attachment to the PLL backbone. In the absence of thrombin, the TBA remains at its secondary conformation. In the presence of thrombin, the TBA folds around the protein and forms a TBA/thrombin complex, acquiring the G-quarter (tertiary) conformation [Fig. 1(d)]. The formation of this complex causes an increase in the effective RI, which changes the optical properties of the guided light.

The sensing devices were incubated with several concentrations of thrombin in the affinity buffer for 1 h. The sensor response was measured in the concentration range from 10 to 100 nM. Between all concentrations steps, the sensing device surfaces were washed with Tris-HCl buffer and the transmission spectra were recorded with the optical spectrum analyzer (OSA) in the measurement buffer. After a complete assay, the sensing probe was washed with 5% hypochlorite solution for 30 min, to regenerate the fiber sensor surface for reuse.

2.4 Fiber-Optic Sensing Setup Assembly

The optical characterization entails the setup shown in Fig. 2 with an OSA or equivalent. The characterization setup for the LPFG [Fig. 2(a)] includes a FS2200 Braggmeter (FiberSensing, SA, Portugal), a fluidic system containing a reaction chamber with capacity of 750 μL, and a laptop (not shown) with LabVIEW software that receives and processes the readout information. The LPFG and FBG sensing elements were characterized using the Braggmeter (working in the 1500 to 1600 nm range with 2.5 pm of resolution) and modified to measure signals both in reflection and in transmission modes.

The setup in Fig. 2(b) for the SPR sensing characterization includes a super luminescent diode source (SLD) model SSFC1550S-A2 (Benchtop SLD—Thorlabs, Germany), a fluidic system where the fiber is fixed, and an OSA (ANDO, model AQ 6315B).

Although the Braggmeter is, by design, capable of simultaneously monitoring two sets of sensors, the SPR sensor at low RI values operates outside its spectral window (1500 to 1600 nm). Therefore, the interrogation of the sensors was done using the OSA and SLD for SPR and the FS2200 SA Braggmeter (for the LPFG). The different setups were used together in order to enable the simultaneous measurements and comparison of the sensor devices.

3 Results and Discussion

3.1 Refractive Index Characterization

Both sensing devices were characterized in terms of the surrounding refractive index (SRI) changes. Solutions with different RI values were prepared by adding ethylene glycol to ultrapure water. Thus, solutions with RI from 1.335 to 1.430 can be obtained varying concentrations of the mixture. In order to accomplish homogeneous solutions, a magnetic stirrer was used and the measurements were accomplished within 5 min after each mixing.

In parallel to the optical characterization of the fiber sensing devices, the RI of the sample solutions was measured at room temperature (26 ± 1°C) using an Abbe refractometer (A. Kruss, Optronic, Germany) with an RI resolution of 2.5 × 10⁻⁴.

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Fig. 2 Scheme of the sensing configurations: (a) long period grating coated with 30 nm of TiO₂ and (b) surface plasmon resonance device in etched single-mode fiber coated with Au and TiO₂.
Solutions with RI in the range from 1.335 to 1.373 were attained.

After each measurement, the sensing device was flushed with 20 ml of IPA and dried with filtered air to avoid contamination to the next sample. Then the spectrum of each sensor was monitored to ensure that the initial spectral conditions were verified.

The characterization of the sensors enables the calculation of the sensor sensitivity through the changes of the spectrum due to changes of the effective SRI.

The wavelength dependence with the external RI of the LPFG and the SPR is presented in Figs. 3 and 4, respectively. The resonance peak of these kind of devices is, in most cases, asymmetric, however, the lower part of the attenuation bands can be fitted by a symmetric curve with reasonable accuracy. To perform this analysis, the minimum optical power of the band was evaluated and then considering only the points 30% above this minimum, a Gaussian function was fitted and all the parameters were extracted, such as the wavelength position and the minimum optical power.

In both sensing devices, the dependence of the wavelength with the RI is nonlinear, nevertheless, a rough linear fitting can be made in the RI range from 1.335 to 1.355 where the corresponding changes induced by the thrombin detection are expected.

Figure 3(a) shows the spectra of the LPFG when placed in different RI solutions. By monitoring the position of the resonant peak as a function of the SRI, it is possible to estimate the spectral sensitivity of the sensor, obtaining the traces presented in Fig. 3(b). Even though the LPFGs are sensitive to temperature, when coupled to the FBG, which is insensitive to the SRI, these variations can be compensated.29 The inset of Fig. 3(b) presents the wavelength shift of the FBG transmission peak showing the thermal variation of each measuring point, which is used to calibrate the LPFG data points leading to a sensitivity of $153.5 \text{ nm RIU}^{-1}$, which is higher than a bare LPFG in the same RI range, as reported in a previous publication.30

In Fig. 4(a), the spectra obtained with the SPR at different SRI liquid samples are presented. Although the shape of the attenuation band is wider, the wavelength shift attained for the same RI samples is more than 20 times higher compared to the LPFG. The data points corresponding to the SPR sensor presented in Fig. 4(b) follow an exponential behavior. However, in the expected working RI range, from 1.335 to 1.355, a linear fit leads to a calculated sensitivity higher than 3000 nm RIU$^{-1}$.
The low temperature variations are not an issue when the SPR device is considered. The effect of temperature using an SPR sensor was discussed previously.\textsuperscript{25} Despite the material properties of the SPR layers being affected by temperature with thermal expansion, these changes are not reflected in the optical properties when such low variations are considered. In addition, it is known\textsuperscript{31} that the RI of liquids change with temperature by a factor of $\sim 1.73 \times 10^{-4}$°C$^{-1}$, which can be neglected for this working range.

### 3.2 Thrombin-Binding Aptamer-Based Optical Fiber Sensing Systems Operation

The sensor performance was tested against varying concentrations of thrombin (10 to 100 nM) in the measuring buffer solution, using both sensing systems, LPFG and SPR-based.

Figure 5(a) displays the spectra of the LPFG coated with 30 nm of TiO$_2$ in series with an FBG across the functionalization procedure with PLL, the immobilization of the DNA probe, the thrombin detection with a concentration of 100 nM, and the regeneration using hypochlorite.

The LPFG response (wavelength shift) to the surface functionalization and thrombin detection measured in buffer solution are shown in Fig. 5(b). Well-defined steps, corresponding to the different layers attached to the LPFG surface, were attained after the thermal effect correction calculated with the FBG.

The PLL coating causes a negative wavelength shift of about 128 pm, which reflects an increase of the effective RI (as reported by Westwood et al. in 2013\textsuperscript{32}). The immobilization of the DNA probe, TBA, causes a resonance peak shift of 165 pm. The binding of TBA-thrombin causes a wavelength shift of about 100 pm. The resolution can be calculated by using the following mathematical equation:

\[
R = \frac{\delta C}{\delta \lambda \sigma},
\]

where $C$ is the thrombin concentration, $\delta \lambda$ is the wavelength shift, and $\sigma$ is the higher standard deviation of the two stages in a certain step. The resolution for this event is about 10.4 nM for a concentration of 100 nM.

It is visible in the regeneration step with hypochlorite that the DNA probe was removed from the sensor leaving the surface completely regenerated and ready for a new functionalization procedure.

The SPR spectra obtained at each step of the thrombin detection are shown in Fig. 6. As previously mentioned, the SPR
The spectrum shape is wider with lower attenuation due to the polarization effect (which is not controlled in this experiment), although with a sensitivity 20 times higher.

The spectral features of the SPR in transmission mode are illustrated in Fig. 7 in terms of the wavelength shift [Fig. 7(a)] and the optical power shift [Fig. 7(b)] of the attenuation band minimum considering the functionalization steps, the thrombin detection, and the regeneration of the sensor. Wavelength shifts of 10.3 and 8.2 nm for the PLL coating and the TBA immobilization, respectively, were achieved. The step corresponding to the thrombin detection shows a variation of 4.94 nm, which is a considerable improvement when compared to \( \sim 100 \) pm from the LPFG results.

The thrombin detection resolutions using the SPR sensor for the wavelength and intensity modulations were calculated as 1.08 and 0.13 nM, respectively.

### 3.3 Sensor Performance to Thrombin Detection

The analytical performance of TBA-based optical fiber sensing systems was investigated by using target thrombin with different concentrations (10, 50, and 100 nM).

The results of the wavelength shift when applying different concentrations of thrombin normalized to the TBA are shown in Fig. 8. The SPR results are represented with empty symbols while the LPFG are represented by the filled ones.

In the SPR configuration, a resonance shift of \( \sim 3.5 \) nm is observed for 10 nM of thrombin. From this data, and considering the system signal-to-noise-ratio, a resolution of 0.54 nM can be calculated. Increasing the concentration to 50 nM leads to a new shift of the resonance of about 1.5 nm, corresponding to a total accumulated shift of 5 nm after the TBA immobilization. A new increase of the concentration to 100 nM results in practically no observable shift. The sensor displays a similar
Table 1 Comparison of the results herein presented with other researchers’ works considering different parameters.

<table>
<thead>
<tr>
<th>References</th>
<th>Allsop et al. 14</th>
<th>Lin et al. 35</th>
<th>Park et al. 36</th>
<th>Razquin et al. 37</th>
<th>This work</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection method</td>
<td>Optical SPR</td>
<td>Optical</td>
<td>Spectroscopic/electrochemical</td>
<td>Optical LMR</td>
<td>Optical SPR/LPFG</td>
</tr>
<tr>
<td>Time for analysis</td>
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<td>80 min</td>
<td>20 min</td>
<td>60 min</td>
<td></td>
</tr>
<tr>
<td>Concentration range</td>
<td>1 nM to 1 μM</td>
<td>250 nM to 1 μM</td>
<td>1 μM</td>
<td>100 nM to 1 μM</td>
<td>10 to 100 nM</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>~1 nM</td>
<td>0.19 μM</td>
<td>—</td>
<td>—</td>
<td>10 nM</td>
</tr>
<tr>
<td>Resolution</td>
<td>50 pM</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.54 nM</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>1 nm/μM</td>
<td>0.04 nm/μM</td>
<td>—</td>
<td>0.04 nm/μM</td>
<td>0.35 nm/μM 0.025 nm/μM</td>
</tr>
<tr>
<td>Regeneration</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

It should be noted that the resonance peak position, which corresponds to the same wavelength shift obtained previously in different trials using only one step between TBA and 100 nM of thrombin. The absence of a wavelength shift from 50 to 100 nM suggests that the sensor reaches a saturation limit. Changes in the PLL/TBA concentrations in the functionalization procedure should give rise to improvement of the thrombin detection efficiency.

In the case of the LPFG, a smaller negative shift is observed. As previously mentioned, the wavelength shift attained for the same RI samples is 20 times higher than that for the SPR compared to the LPFG, as confirmed by the data. The lower sensitivity to the external RI shown by the LPFG was found to be a limitation to a proper quantification of the thrombin with accuracy. Nevertheless, it validated the working principle also with LPFG. Furthermore, several strategies can be explored to greatly enhance the sensitivity of LPFG-based devices that could enable quantification.

Although the comparison between these two system devices can be achieved by the introduction of a figure of merit (FOM) by using the following equation:

\[
FOM = \frac{S}{FWHM}.
\]

which is defined by the ratio between the sensitivity to the external RI (S) and the full width at half maximum. Considering the sensitivities to the external medium previously calculated as 3143 nm/RIU and 204 nm/RIU for the SPR and LPFG sensors, respectively, the calculated FOM results in 26.2 and 18.3, which is an indicator of a better performance of the SPR configuration even with a wider spectral shape.

These results are comparable to other researchers’ work, as can be observed in Table 1.

This comparison is made through the same recognition method using a DNA aptamer and it shows indicators of good performance considering the sensitivity to the thrombin detection and the total regeneration of the sensor surface.

3.4 Cross-Reactivity to Other Molecules

The TBA was already presented as an aptamer that shows good affinity and specificity against thrombin26 and does not interact with other plasma proteins or thrombin analogues such as gamma-thrombin;27 nevertheless, its cross-reactivity with other molecules was experimentally tested.

To assess the nonspecific binding of other molecules that could be present in real samples of the different sensor configurations, the LPFG and SPR sensing devices were incubated with 100 nM of human serum albumin (HSA) for 60 min in the same affinity buffer. Because HSA is the most abundant protein in human plasma and is a potential interference present in real blood or serum samples, it is a suitable protein to be tested in the present work.

In the case of the LPFG, the wavelength shift obtained has an insignificant value (~0.022 nm), which was found to be below the measurement error. In the SPR configuration, a higher shift of about 0.9 nm was observed (data not shown), for 100 nM of HAS, however, this is a small fraction of the shift obtained when incubating with only 10 nM thrombin.

These results show good chemical and biological specificity of TBA binding to thrombin even in the presence of potential interferences.

4 Conclusions

The detection of thrombin was compared using two different optical fiber sensing configurations, LPFG- and SPR-based. LPFG with titanium dioxide coating improves the sensitivity to external refractive indices and has proven the feasibility of thrombin detection. Despite the temperature dependence, the robustness and the narrow bands lead to possible applications with multiplexing capabilities. The coupling to FBGs allows the postprocessing corrections to thermal variations. The lower sensitivity to the external RI is a barrier to quantify the thrombin concentrations with accuracy. Nevertheless, it can be used to act for presence/absence detection.

The higher RI sensitivity of the SPR with a higher FOM when compared to the LPFG response and the insensitivity to thermal variations have shown detection levels of thrombin down to 10 nM. The regeneration of the surface was attained in both configurations allowing a new functionalization procedure and, therefore, the reuse of the sensing system.

Both refractometric platforms allow to use the external RI variations to quantify thrombin concentration in a buffer solution, therefore, the same concept could, in principle, be applied to estimate the RI/thickness of the PLL layer or even to quantify the amount of the TBA immobilization.

Acknowledgments

This work is financed by the North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL
2020 Partnership Agreement, and through the European Regional Development Fund (ERDF) within projects POCI-01-0145-FEDER-006961 and Coral - Sustainable Ocean Exploitation: Tools and Sensors/NORTE-01-0145-FEDER-000036. L.C. acknowledges the support from FCT grant SFRH/BD/78149/2011.

References


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