

Fiber-based label-free D-dimer detection for early diagnosis of venous thromboembolism

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ABSTRACT

D-dimer is a useful diagnostic biomarker for deep vein thrombosis or pulmonary embolism, collectively referred to as venous thromboembolism (VTE). The ability to detect in real-time the amount of D-dimer with a fast and reliable method is a key step to anticipate the appearance of these diseases. The combination of fiber-optic-based platforms for biosensing with the nanotechnologies is opening up the chance for the development of *in situ*, portable, lightweight, versatile, reliable and high-performance optical sensing devices towards lab-on-fiber technology. The generation of lossy mode resonances (LMRs) by means of the deposition of nm-thick absorbing metal-oxide films on special geometric-modified fibers allows measuring precisely and accurately surface refractive index changes, which are due to the binding interaction between a biological recognition element and the analyte under investigation. This approach enhances the light-matter interaction in a strong way, thus turning out to be more sensitive compared to other optical technology platforms, such as fiber gratings or surface plasmon resonance. Here, the results of a highly specific and sensitive biosensor for the detection of D-dimer based on LMR in fiber-optics are presented by monitoring in real-time the shift of the LMR related to the biomolecule interactions thanks to a conventional wavelength-interrogation system and an ad-hoc developed microfluidics. A detection limit of 100 ng/mL, a value 5-fold below the clinical cutoff value, has been attained for D-dimer spiked in human serum. The comparison of the results achieved with proteomics-based methodologies, which allows for the identification of beta- and gamma-chains of fibrinogen, demonstrates the ability of our platform to specifically (>90%) recognize D-dimer.

Keywords: venous thromboembolism, D-dimer, label-free, optical fiber biosensor, lossy mode resonance

1. INTRODUCTION

D-dimer antigen, the smallest product originated from cross-linked fibrin degradation, is used, above a cutoff value (0.5 µg/mL), as a biomarker for deep vein thrombosis or pulmonary embolism, collectively referred to as venous thromboembolism (VTE) [1]. With about 10 million cases of VTE occurring every year, it represents the third leading vascular disease after acute myocardial infarction and stroke. Clinical routine of suspected VTE includes the sequential application of a clinical decision rule and D-dimer testing [2] with assays used to predict which patients are more likely to suffer recurrent thrombosis when anticoagulant treatments are stopped [3]. Therefore, the ability to detect in real-time the amount of D-dimer with a fast and reliable method is a key step to anticipate the appearance of these diseases.

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The combination of fiber-optic-based platforms for biosensing with the nanotechnologies is opening up the chance for the development of *in situ*, portable, lightweight, versatile, reliable and high-performance optical sensing devices towards lab-on-fiber technology [4,5]. One of the most widespread phenomenon related to the use of thin films for sensing is surface plasmon resonance (SPR), localized SPR [6], Bloch surface wave [7] and fiber gratings [8,9]. Recently, the phenomenon of guided mode resonance was also applied to fiber-optic, under the name of lossy mode resonance (LMR) [10]. The generation of lossy mode resonances (LMRs) by means of the deposition of nm-thick absorbing metal-oxide films on special geometric-modified fibers allows measuring precisely and accurately surface refractive index (RI) changes, which are due to the binding interaction between a biological recognition element also known as receptor and the analyte under investigation. This approach enhances the light-matter interaction in a strong way, thus turning out to be more sensitive compared to other optical technology platforms and, outstandingly, attaining the detection of femtomolar concentrations in real samples of human serum [11].

Here, by making use of the previous approach, the results of a highly specific and sensitive biosensor for the detection of D-dimer based on LMR in fiber-optics are presented by monitoring in real-time the shift of the LMR (see Figure 1a) as a function of the biomolecule interactions (D-dimer antibody and D-dimer protein) as shown in Figure 1b. The LMR-based fiber-optics platform consists also of a conventional wavelength-interrogation system and of an ad-hoc designed microfluidics. Different metal-oxide materials can be used to excite LMR, showing similar RI sensitivity, except for tin oxide (SnO_{2-x}) which exhibits improved performance. From the calibration curves obtained, a detection limit of 100 ng/mL, a value 5-fold below the clinical cutoff value, has been attained for D-dimer spiked in human serum samples. The comparison of the results achieved with gel electrophoresis and Western-blotting (WB) methodologies (proteomics), which allows for the identification of beta- and gamma-chains of fibrinogen, demonstrates the ability of the proposed platform to specifically (>90%) recognize D-dimer. Therefore, this technology potentially represents a paradigm shift in the development of a simple, high-specificity and label-free biosensing platform, which can be applied to speed up diagnostic healthcare processes of VTE for an early diagnosis and personalized medicine.

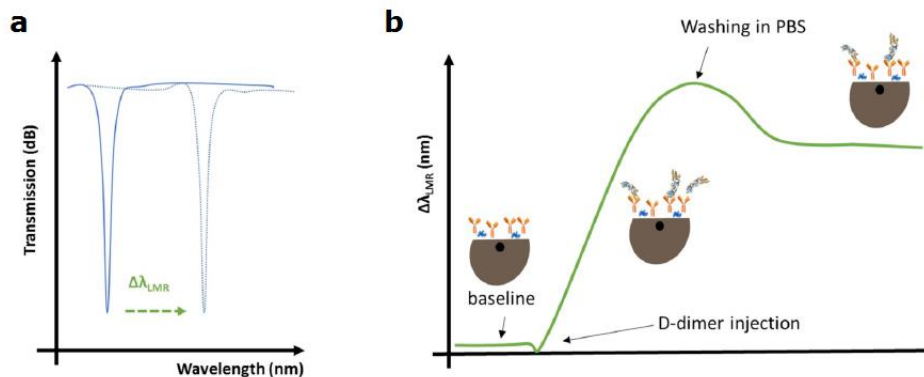


Figure 1. (a) Schematic illustration of the sensing mechanism based on the resonance shift in the transmission spectrum related to surface RI changes. (b) Schematic representation of the LMR shift due to a specific binding interaction occurring along the functionalized fiber surface between the biological recognition element (capture antibody; anti-D-dimer) and the analyte under investigation (antigen; D-dimer).

2. MATERIALS AND METHODS

2.1 Experimental setup and D-dimer assay protocol

The proposed optical sensing system is based on the lossy mode resonance, a technology based on enhancement of the evanescent field of the modes guided in the optical fiber due to the guidance of a mode in a nanometric thin-film. This overlay presents the real part of the RI higher than that of the fiber substrate and the imaginary part low but not negligible [10]. Moreover, the sensitivity of LMR-based devices can be improved if the RI of the material deposited on the fiber is as high as possible [10]. In addition, the optical platform is critical for the performance of the biosensing system: if a side-polished fiber is used instead of a cladding removed multimode fiber, the performance can be greatly improved [11]. This justifies the choice of SnO_{2-x} as coating material together with a D-shaped fiber.

In order to obtain the D-shaped fiber configuration, standard single mode fibers (Corning SMF-28) with a cladding/core diameter of 125/ 8.2 μm are used. 17 mm of the fiber are progressively polished down until an attenuation of 1 dB at 1550 nm is attained in high RI matching oil (1.5 RIU). Concerning the procedure of coating deposition of the SnO_{2-x} thin film, the D-shaped fiber is placed inside a DC sputter machine (K675XD, Quorum Technologies) at a partial pressure of argon of 9×10^{-2} mbar and an intensity of 90 mA, attaining a thickness of around (160 ± 10) nm [11]. After the deposition process, the fiber sensor is located inside a thermo-stabilized microfluidic system [9]. This allows for control and manipulation of small volumes of liquid samples as well as temperature stabilization. Figure 2a details the entire microfluidic system which characteristics are provided in the literature [12].

The sketch of the LMR-based D-shaped fiber biosensor is displayed in Fig. 2b. After the fiber surface coating with SnO_{2-x} , the anti-D-dimer receptor is grafted on the sensor surface, according to the steps precisely described in the recent literature [12]. Afterwards, the assay is completed by injecting different solutions of D-dimer protein at increasing concentrations that range from 100 ng/mL up to 100 $\mu\text{g/mL}$ for 15 min each at a flow rate of 7 $\mu\text{L/min}$. A washing step with PBS is then carried out for 5 min at 20 $\mu\text{L/min}$ in order to measure the residual shift of the LMR signal due to the receptor-analyte binding interaction, and hence to discard any volume effect related to the solution RI [13]. In the first experiment when D-dimer is spiked in PBS running buffer, selectivity is confirmed by injecting a solution of C-reactive protein, which is another biomarker for heart diseases like stroke. Conversely, in the next experiments, D-dimer is spiked in diluted (1:10 (v/v) in PBS) and undiluted human serum matrices as realistic selectivity test.

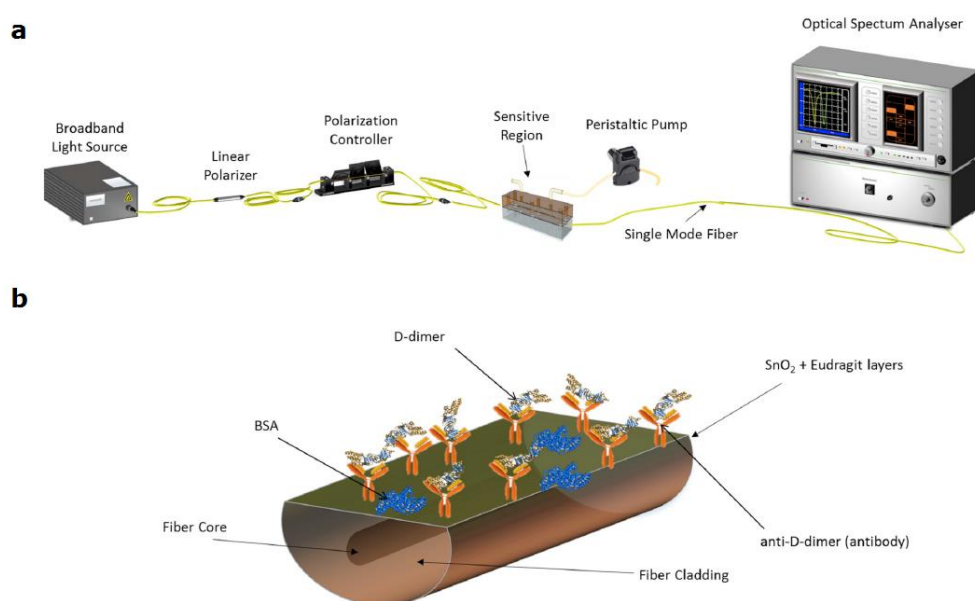


Figure 2. (a) Schematic illustration of the entire experimental setup for the detection of D-dimer together with the microfluidic system. (b) Schematic representation of the LMR-based D-shaped fiber biosensor with the biomolecules involved.

2.2 Comparative analysis: gel electrophoresis and Western-blotting

The entire technical workflow, which is used to verify the binding of D-dimer onto the functionalized biosensor surface, is detailed in the recent literature [12]. To sum up, protein denaturation from the fiber surface is firstly performed by applying a lysis buffer containing urea (7M), thiourea (2M) and DTT (0.5%) for 5 min at 95 $^{\circ}\text{C}$. Afterwards, protein content is let it precipitate by acetone at -20 $^{\circ}\text{C}$. Later, both D-dimer protein and anti-D-dimer antibody (1–5 μg) are re-suspended in 4x *Laemmli* buffer in the presence of betamercaptoethanol. After that, the biomolecules are separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a concentration of acrylamide of 12.5%. Finally, anti-D-dimer antibodies are used to detect potential D-dimer products by WB using the procedure described in the literature [14]. Briefly, D-dimer protein is re-solved in 12.5% acrylamide gels and electrophoretically transferred onto nitrocellulose membranes using a Trans-blot Turbo transfer system in 7 min (25 V). Membranes are then probed with the antibody at 1:1000 dilution in 5% non-fat milk. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000 dilution), the immunoreactivity is visualized by enhanced chemiluminescence (PerkinElmer)

using a Chemidoc MP Imaging System (Bio-Rad). Finally, densitometric analysis is performed with the Image Lab tool (Software Version 5.2).

3. RESULTS AND DISCUSSION

In a previous work [11], it was demonstrated that the use of SnO_{2-x} as thin film to excite LMR is better than indium tin oxide (ITO), since better performances were attained in terms of both refractive index sensitivity and limit of detection. Moreover, other metal-oxide absorbing materials were explored to excite LMR and tested as optical refractometers, such as amorphous silicon, indium gallium zinc oxide (IGZO) and zinc oxide (ZnO) [12]. However, they accounted for RI sensitivities that are comparable or lower than ITO. In addition, even though a material with a higher sensitivity is used, its behavior in a microfluidic system should be taken into account. By comparing the response of different materials in the same fluidic condition, it turns out that SnO_{2-x} experienced the greater stability, thus reinforcing the idea of using tin oxide as the best thin film [12].

According to the assay protocol sketched in Section 2 and as detailed in the literature [12], D-dimer was detected in a label-free approach by spiking it into three different running buffers: phosphate buffered saline (PBS 1X, pH 7.4), PBS-diluted (1:10) human serum and undiluted human serum.

The ability of the nanometric film coated D-shaped fiber biosensors is firstly proved with the detection of D-dimer at different concentrations in PBS. The transmission spectrum of the sensor has been monitored during the whole experiment and the wavelength shift of the LMR has been tracked in real time, thus achieving the related sensorgram where the signal change, compared to the baseline, is displayed as a function of the injection of D-dimer solutions ranging from 100 ng/mL up to 100 $\mu\text{g/mL}$. What has been observed is that the LMR wavelength shifts toward longer wavelengths as the concentration of D-dimer increases. Moreover, it is worth pointing out that, as it occurs in all label-free biosensors, after the injection of the target analyte at different concentrations and the subsequent achievement of a steady-state condition, a washing step with the running buffer is performed in order to remove all the not-bound D-dimer. This means that the wavelength shift after the washing step with respect to the signal value before the D-dimer solution injection, commonly named residual shift, is only related to the amount of D-dimer seized by the specific receptor grafted on the fiber surface, which in turn is directly related to the D-dimer concentration [13]. Figure 3(a) shows the calibration curve of the device, which reports the shift of the LMR wavelength as a function of the D-dimer concentration. These results have been achieved by repeating the same assay protocol while using four different D-shaped fiber biosensors ($n = 4$). Therefore, each experimental point expressed in terms of the mean value and the respective standard deviation (black error bars) contains the information of the repeatability and reproducibility of the whole optical system and the biochemical protocol [13]. As shown in Figure 3(a), the response of the device exhibits the typical sigmoidal behavior without reaching the upper asymptote where the saturation of the biosensing layer is attained, because small concentrations of D-dimer have been investigated. The experimental points are fitted by the Hill function (red curve), which is formally equivalent to the Langmuir isotherm, a well-accepted mathematical model that provides a way in which to quantify the degree of interaction between a receptor and an analyte. In addition, the calibration curve allows extrapolating the limit of detection (LOD) of the biosensor, defined as the signal of the blank plus three times the standard deviation of the blank [13]. By looking at the inset of Figure 3(a), the LOD is 10 ng/mL (50 pM), considering a standard deviation of the blank of 16.8 pM (picometer).

The next step was the development of the same assay protocol using samples of human serum in order to assess the application of the biosensor as much as close to real clinical settings. Figure 3(b) details the calibration curve of the two biosensors when the assays have been performed with diluted (blue symbols) and undiluted (green symbols) solutions of human serum. The value of each experimental point refers to the average of 15–20 distinct measurements at flow stopped after each washing step in PBS. Again, the red curves detail the sigmoidal response fitted by the Hill function. The LODs are 100 ng/mL (500 pM) in the case of diluted serum, considering a standard deviation of the blank of 34 pM (picometer), and 320 ng/mL (1.6 nM) in the case of undiluted serum, respectively. In the latter case, when undiluted serum was used, a higher LOD has been attained and the reason stems from a greater difficulty, and hence a less probability for the D-dimer to reach the receptor due to a denser matrix and/or to a more intense electric charge effect over the biomolecules [15]. However, it is clear that the proposed device is able to detect D-dimer below the clinical cutoff value of 500 ng/mL, even in routinely-used clinical settings. As the biosensor specificity is concerned, C-reactive protein was used in the first case when the analyte was spiked in PBS, whilst human serum directly acted as negative control in the second and third cases. It was recently proved that a specificity greater than 90% has been attained [12].

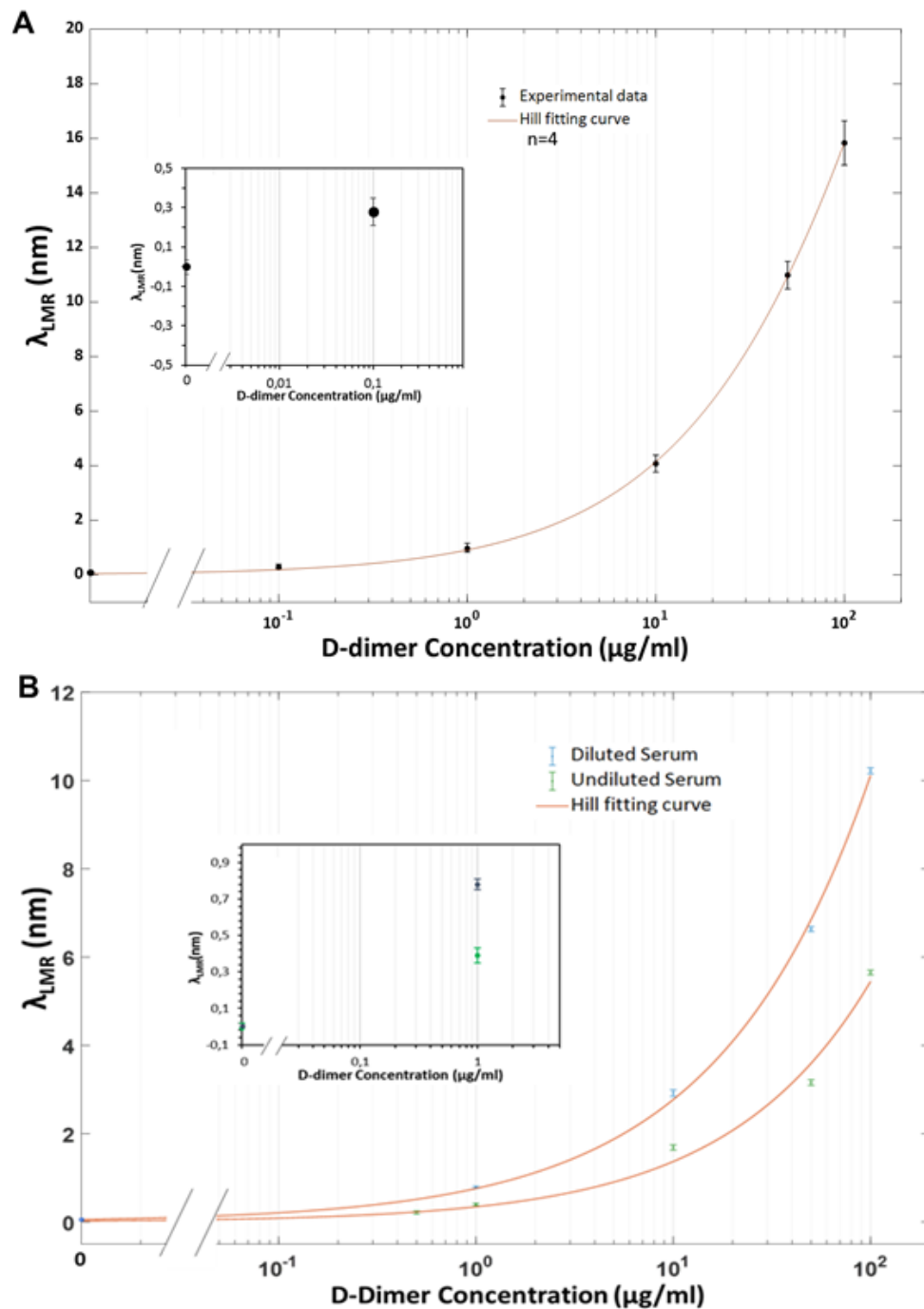


Figure 3. (a) Calibration curve obtained from distinct ($n = 4$) D-shaped fiber biosensors detecting D-dimer in PBS as running buffer. (b) Calibration curves obtained from two D-shaped fiber biosensors detecting D-dimer in 1:10 diluted (blue) and undiluted (green) human serum solutions. The red curves detail the fitting curves of the experimental data using the Hill sigmoidal function.

In order to assess and validate the binding of D-dimer onto the functionalized fiber surface as a comparative analysis, a technical workflow has been elaborated and is shown in Figure 4(a). It consists of the detachment of D-dimer from the fiber piece, then of its separation through SDS-PAGE and, finally, of its detection by Western-blotting. All the steps are

detailed in the literature [12]. Figure 4(b,left) shows the results of D-dimer separation by SDS-PAGE using the Ponceau staining, while on the right it details the results attained with WB. It is clear that Ponceau staining approach is not sensitive enough to detect the presence of D-dimer in fiber (2nd column), but is useful because it does not appear to have a deleterious effect on the sequencing of blotted biomolecules (3rd column), and hence was the selected method for locating them through WB. In fact, as it can be observed from Figure 4(b,right), WB exhibits a specific band of D-dimer detached from the fiber surface and placed between 25 kDa and 35 kDa (2nd column), thus confirming the effectiveness and reliability of the proposed device.

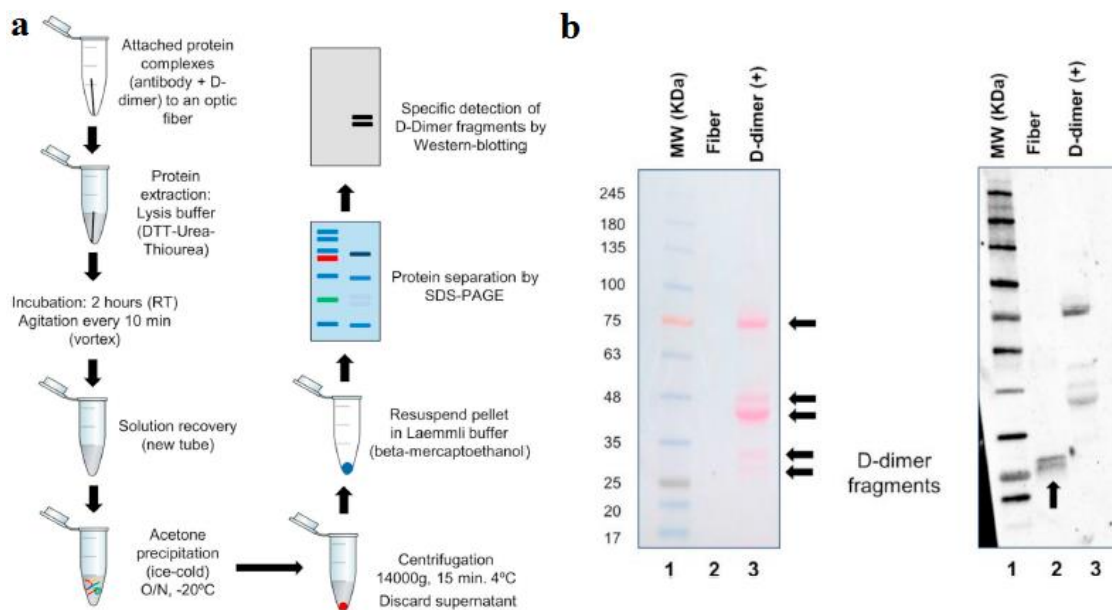


Figure 4. (a) Technical workflow used to verify the binding of D-dimer onto the functionalized surface of the D-shaped fiber biosensor. (b) Results of D-dimer separation by SDS-PAGE using the Ponceau staining (left) and the comparison through Western-blotting when D-dimer is detached from the functionalized fiber surface (right).

4. CONCLUSIONS

This paper analyzes the sensing performance and feasibility of LMR-based optical biosensors realized by means of SnO_{2-x} nanocoated D-shaped fibers for the specific detection of D-dimer/anti-D-dimer interactions in a label-free approach. The attained results proved that the proposed device is able to detect D-dimer with a LOD below the clinical cutoff value of 500 ng/mL, not only in simple matrix as PBS but also in routinely-used clinical settings as human serum. Therefore, this technology potentially represents a paradigm shift in the development of a simple, high-specificity and label-free biosensing platform, which can be applied to speed up diagnostic healthcare processes of VTE for early diagnosis and personalized medicine.

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