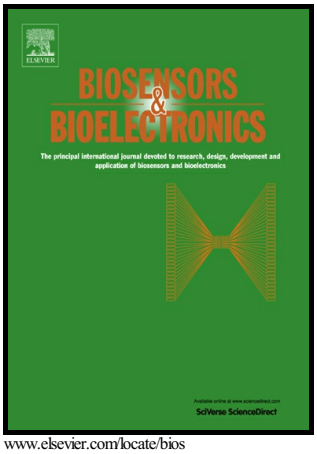

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Rapid and ultrasensitive detection of active thrombin based on the Vmh2 hydrophobin fused to a Green Fluorescent Protein

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Abstract

A fusion protein designed in order to combine the fluorescence emission of the Green Fluorescent Protein (GFP) with the adhesion ability of the class I hydrophobin Vmh2 was heterologously produced in the yeast *Pichia pastoris*. The Vmh2-GFP fusion protein has proven to be a smart and effective tool for the study of Vmh2 self-assembling.

Since the two proteins were linked by the specific cutting site of the thrombin, the fusion protein was used as the active biological element in the realization of a thrombin biosensor. When the thrombin present in the target solution specifically hydrolyzed its cleavage sequence, a consequent decrease in the fluorescence intensity of the sample could be observed. The Vmh2-GFP based assay allowed quantification of thrombin in solution with a detection limit of 2.27 aM. The specificity of the assay with respect to other proteases and proteins granted the measurement of thrombin added to healthy human plasma with same high sensitivity and a limit of detection of 2.3 aM. Further advantages of the developed biosensor are the simplicity of its design and preparation, and the low requirements in terms of samples, reagents and time.

Keywords:

Class I hydrophobin; amyloid fibrils; yeast heterologous expression; protease

1 Introduction

Thrombin is a crucial enzyme in blood coagulation and is an analyte which has been the subject of increasing interest in the last few years. This serine protease can directly transform soluble fibrinogen into insoluble fibrin, and can facilitate blood hemostasis (Hsieh 1997). If the level of thrombin in the body is too high, thromboembolic diseases or even death will result, while a low amount of thrombin will induce an excessive bleeding. During the coagulation process, its concentration varies considerably, even from nM to mM (Na et al. 2015). The development of rapid, sensitive and accurate thrombin detection in real-time is essential to adjust the strategies of drug administration for certain patients with severe thrombin-related medical problems. Moreover, monitoring thrombin is of great importance in the early diagnosis of some types of disease, such as hemorrhagic or thromboembolic complications and Alzheimer's disease (Chen et al. 2016), and as a useful marker in the diagnosis of pulmonary metastasis (Hernandez-Rodriguez et al.1997). Numerous methods of thrombin detection have been proposed since the beginning of this century. Most of them have been based on the use of thrombin binding aptamers, which are promising candidates as sensing elements for the development of real-time biosensors. The molecular recognition between thrombin and aptamers and/or the biosensor configuration itself have been reported in several of these studies, but as a proof-of-concept rather than as an instrument in the development of a thrombin detection method (Xu et al. 2009; Qin et al. 2015; Derkus 2016). Different methods have been proposed, including an optical approach (Yan et al. 2011), electrochemiluminescence (Wang et al. 2011; Lu et al. 2015), electrochemical impedance spectroscopy (Lu et al. 2015; Derkus 2016), an electrochemical approach (Zhao et al. 2011; Xu et al. 2013), colorimetry (Liang et al. 2011), surface plasmon resonance (Yu et al. 2004; Jalit et al. 2013; Trapaidze et al. 2016), and quartz crystal microbalance with dissipation monitoring (Chen et al. 2010). Wide ranges in the Limit of Detection (LOD), spanning from nM (10^{-9} M) (Trapaidze et

al. 2016; Kuang et al. 2016) down to fM (10^{-15} M) have been reported (Zhao and Gao 2015; Heydari-Bafrooel et al. 2016). A higher sensitivity (aM, 10^{-18}) has been obtained through modifications with fluorophores (Yu et al. 2004) and the use of electrochemical impedance spectroscopy (Lu et al. 2015).

In this study, a novel method to reliably quantify the thrombin concentration has been developed based on the detection of thrombin effectively active in the sample. The biological element, able to detect this protease enzyme in the biosensor system proposed, was designed in order to combine the fluorescence of the Green Fluorescent Protein (GFP) with the adhesion ability of the class I hydrophobin Vmh2, linked by the specific cutting site (LVPR/GS) of the thrombin. The designed fusion protein was heterologously produced in the yeast *Pichia pastoris*. The sensing mechanism is that the thrombin present in the target solution specifically recognizes and hydrolyzes its cleavage sequence with a consequent decrease in the fluorescence intensity of the sample.

Fluorescent Proteins (FPs) are genetically encoded fluorophores, which can be easily used as fluorescent probes for the tracking of proteins, organelles or whole cells, and as the basis for the construction of biosensors (Enterina et al. 2015). Protein engineering (Chen et al. 2012) and the discovery of new FPs (Mishin et al. 2015) have expanded the possibilities of use of these biosensors. Other emerging technologies to efficiently exploit FPs include different structural approaches aimed at turning on or shifting the fluorescence emission in response to environmental changes (Enterina et al. 2015).

Class I hydrophobins are small proteins able to self-assemble into amphiphilic films at hydrophobic-hydrophilic interfaces (Linder 2009). They efficiently adhere to different hydrophobic surfaces, such as teflon (Askolin et al. 2006), polystyrene (Wang et al. 2010), silicon (De Stefano et al. 2007, 2009) and graphene (Gravagnuolo et al. 2015), forming extremely stable films that can be dissolved only in strong acids (e.g. 100% trifluoroacetic acid, TFA). Class I hydrophobins form fibrillar structures, known as rodlets, showing many structural analogies with amyloid fibrils. Hydrophobin assemblies represent a versatile and simple method for the immobilization of proteins

(De Stefano et al. 2009; Wang et al. 2010; Sun et al. 2011; Longobardi et al. 2015; Zhao et al. 2007) on diverse substrates for the development of several biotools such as biosensors (Qin et al. 2007; Zhao et al. 2009; Wang et al. 2010; Sun et al. 2011; Piscitelli et al. 2016). The class I hydrophobin Vmh2 from the basidiomycete fungus *Pleurotus ostreatus* is able to spontaneously form stable and homogeneous layers on hydrophilic or hydrophobic surfaces, changing their wettability (De Stefano 2007), and has been efficiently exploited for several different applications (Longobardi et al. 2015; Gravagnuolo et al. 2015; Piscitelli et al. 2016).

The fluorescent assay developed in this study is a promising alternative approach compared to the analytical methods currently used to detect thrombin in the plasma. In fact, it allows a reliable and sensitive measurement of the analyte of interest in less than 20 minutes and without the need for specialized personnel, costly equipment or an excessive amount and manipulation of blood.

1. Materials and Methods

2.1 Materials

Reagents were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Expression vectors and yeast strains were purchased from Biogrammatics, Ltd (Las Palmas Dr, Carlsbad, CA, USA). DNA restriction and modifying enzymes were supplied by Promega (Madison, WI, USA). The fusion protein encoding gene was synthesized by Thermo Fischer Scientific (Waltham, Massachusetts, USA). Culture media were bought from BD Difco (Becton Drive, Franklin Lakes, NJ USA). The human factor Xa was purchased from New England Biolabs (Ipswich, MA, USA).

2.2 Vector construction

A synthetic gene encoding for the GFP from *Aequorea victoria* in frame with the thrombin cleavage site and with Vmh2 from *P. ostreatus* was designed (1008 bp) and optimized according to *P. pastoris* codon usage. The gene product was restricted with *BsaI* and ligated into the corresponding site of the pJGG_αkR vector in-frame with the α-factor (signal peptide) under the control of the constitutive GAP promoter, yielding the recombinant plasmid pJGG_Vmh2-GFP. The recombinant plasmid was linearized by *BsiW*I and transformed into *P. pastoris* BG-10.

2.3 Transformation of *P. pastoris* and screening of the clones

P. pastoris cells were collected from a solid culture and grown in 50 ml of YPD medium (10 g l^{-1} yeast extract, 20 g l^{-1} bacto tryptone, 20 g l^{-1} glucose) at 28°C on a rotary shaker (150 rpm) over night. The overnight culture was diluted 1:5,000 in fresh medium and grown overnight up to 1.3-1.5 OD_{600}. Next, the cells were sedimented by centrifugation at 2,000 rpm at 4°C for 5 min, resuspended in YPD, 0.02M Hepes, 0.04 M DTT, and shaken at 30°C for 25 min at 100 rpm. Then the cell suspension was diluted 1:10 with cold H_2O and the cellular pellet washed with cold H_2O, and cold 1 M sorbitol. The cell pellet was then resuspendend in 1M sorbitol and mixed with the linearized transforming DNA plasmid (up to 0.5 μg). *P. pastoris* transformation was performed by electroporation with a Bio-Rad Micro-Pulser apparatus (BioRad, Hercules, California, USA), as specified by the manufacturer. Immediately afterwards, 1 mL of Recovery Media (1:1

YPD:sorbitol) was added to the electroporation cuvette and the whole content was transferred to a sterile tube. The tube was then incubated at 28°C for 3 hours under shaking conditions. The cell suspension was spread on YPD plates containing 900 μg mL^{-1} of G418 and incubated for 3-7 days at 28°C until colony formation. The colonies were collected, inoculated in Minimal Dextrose Medium (MD) (13 g l^{-1} Yeast Nitrogen Base with ammonium sulfate w/o amminoacids, $4x10^{-4}$ g l^{-1} biotin, 20 g l^{-1} glucose), and grown at 28° C on a rotary shaker (250 rpm). Aliquots were daily withdrawn and assayed for cell density and for GFP fluorescence emission using the Synergy H4 (Biotek Instruments, Winooski, Vermont, USA) acquiring data through top-reading mode. The excitation wavelength was fixed at 450 nm and emission spectra were recorded between 460 and 560 nm. The emission spectra were then corrected by subtracting the blank (the supernatant of the wild-type *P. pastoris* strain).

2.4 Expression and purification of the fusion protein Vmh2-GFP

Pre-inoculum preparation of the recombinant selected clone was performed at 28°C overnight in MD. The pre-inoculum was diluted 1:100 in the same medium. 10 g l^{-1} Yeast Nitrogen Base (YNB) was re-added to the medium when the culture reached the stationary phase. The recombinant protein was recovered after 8 days and the cells were removed by centrifuging for 20 minutes at 7,000 rpm at 4°C. The supernatant was recovered, concentrated and dialyzed towards 50 mM Tris-HCl buffer, pH 8.0, using Centricon Centrifugal Filter Units with Ultracel YM-10 membrane (Merck, Darmstadt, Germany). The protein concentration was determined using the Bradford method (BioRad) according to the manufacturer's instructions and using BSA as the standard.

2.5 PAGE analyses

The purity of the sample and the Mw of the protein were verified by 15% SDS-PAGE, stained with Coomassie brilliant blue R-250.

Semi-denaturing PAGE was performed in the same conditions except for the reduction and heat denaturation of the samples before loading. The fluorescent protein band was visualized by

exposing the gel to UV light by means of an UV transilluminator (Vilber Lourmat, Eberhardzell, Germany).

2.6 Contact angle measurement

Contact angle measurements were performed by using a KSV Instruments LTD CAM 200 Optical Contact Angle Meter: each contact angle was calculated as the average of the values obtained from three drops having the same volume (about 2 µL of a 0.1 mg mL^{-1} protein sample), spotted on different points of a polystyrene surface, air dried and washed three times with buffer.

2.7 Thioflavin assay

Vmh2-GFP (from 0.1 to 1 mg mL^{-1}) in 50 mM Tris HCl buffer, pH 8.0, was immobilized in multiwell plates (described in paragraph 2.9) and washed three times with the same buffer. 100 µL of 30 µM thioflavin (ThT) was added to the wells and incubated at room temperature for 5 minutes. The ThT fluorescence intensity of each sample was recorded using 435 nm excitation.

2.8 Fluorescence microscopy

Fluorescence analysis was performed by means of a Leica Z16 APO fluorescence macroscope equipped with a Leica DFC300 camera (Leica, Wetzlar, Germany). A I3 filter was used for the image acquisition, consisting of a 450-490 nm band-pass excitation filter, a 510 nm dichromatic mirror, and a 515 nm suppression filter.

Confocal microscopy was performed by means of a Carl Zeiss LSM 700 apparatus using the tunable Argon 488 nm laser at 90.0% (Carl Zeiss, Oberkochen, Germany). The excitation and emission measurements for the Vmh2-GFP images were 488 and 518 nm, respectively.

2.9 Vmh2-GFP immobilization on multiwell plates

Several tests of immobilization of 100 µL of the Vmh2-GFP solutions on polystyrene multiwell plates were carried out, changing different parameters: the protein concentration, the presence of a preformed layer of native Vmh2, and the mixing of the fusion protein with the native Vmh2 (0.1 mg mL^{-1} final concentration).

After incubation at 28° C overnight, removal of the residual liquid and three washes with 50 mM Tris HCl buffer, pH 8.0 to eliminate any unbound proteins, the fluorescence of the GFP was measured in the same buffer. The immobilization yield in terms of fluorescence was calculated considering the immobilized fluorescence with respect to that incubated in the well. To assess the yield in terms of protein amount, the immobilized content was calculated as the difference between the quantity used for the immobilization and that measured in the residual liquid and in the washings.

2.10 Thrombin assay

Defined volumes (100 µL) of thrombin protease at increasing concentrations (from 450 aM to 4.5 µM) in Tris-HCl 50 mM, pH8 were added to the Vmh2-GFP coated wells and incubated at 37°C for 15 minutes. After two washes with 50 mM Tris-HCl buffer, pH 8.0, to remove the hydrolyzed proteins, the remaining fluorescence was measured in the same buffer.

Solutions of thrombin at varying concentrations were also added to human plasma to test the performance of the sensor in complex matrixes. Human plasma samples were collected in a citrate anti-coagulated tube from healthy subjects. Fibrinogen was removed through precipitation according to the method reported in the literature (Bini et al. 2007). The protein amount of the raw plasma and of the eluted solution was evaluated by spectrophotometric measurements at 280 nm and a loss of protein content (40 %) was detected after precipitation of fibrinogen.

To promote the transformation from prothrombin to thrombin, 8 nM human factor Xa in the presence of $CaCl_2$ (0.03 M) were added to the plasma.

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2. Results and discussion

3.1 Production and characterization of the fusion Vmh2-GFP protein

The recombinant expression system developed in this work allowed for the production and secretion in *P. pastoris* of the class I hydrophobin Vmh2 from *P. ostreatus* fused to the enhanced GFP from *A. victoria*. The secreted fusion protein Vmh2-GFP was found to be homogeneous after only two steps, concentration and dialysis, and the obtained yield was about 50 mg L^{-1}. SDS PAGE analysis displayed the presence of a unique protein band at the expected molecular weight (Fig. S1A). The functionality of the GFP moiety was confirmed both by the green fluorescence exhibited by the protein band on partially denaturing SDS-PAGE (Fig. S1B) and by the emission spectra registered for the purified protein (Fig. S1C).

With the aim of verifying the behavior of the Vmh2 moiety within the chimeric protein, its ability to reach the hydrophobic-hydrophilic interfaces, to adhere to the surface and to form amyloid-like fibrils was investigated. Bubble formation was induced by insufflating air in the protein solution, after which a 70-80% reduction of fluorescence in the solution was measured. The obtained foam was deposited on microscopy glasses, heat-dried, and analyzed through fluorescence microscopy. Bubble images clearly revealed the presence of the protein at the air-water interfaces (Fig. 1A).

The ability of the Vmh2-GFP protein to adhere to the hydrophobic polystyrene surface, thus modifying its wettability, was monitored by water contact angle measurements after protein deposition and washing. The polystyrene surface, strongly hydrophobic in nature, became clearly hydrophilic (Fig. 1B) and the effect of Vmh2-GFP was more evident than that of native Vmh2, due to the higher hydrophilicity of the protein fused to Vmh2.

FIGURE 1 PROTEIN CHARACTERIZATION

The formation of amyloid-like structures on the polystyrene surface was verified through Thioflavin T assay. The higher were the protein concentrations, the greater was the fluorescence intensity due to the presence of fibril-ThT complexes (Fig. 1C). Fluorescence microscope images of the fusion

protein deposited on the polystyrene surface at different concentrations showed the presence of protein aggregates at all the tested concentrations, while fluorescent fibrils were detectable at protein concentrations higher than 0.05 mg mL^{-1} (Fig. 1D).

These results highlighted the finding that the Vmh2-GFP fusion protein might be a smart and effective tool for the study of Vmh2 self-assembling. Moreover, the developed recombinant expression system could allow the production and secretion of other Vmh2-fused proteins useful for the direct functionalization of different surfaces, thus expanding the range of applicability of these "self-immobilizing" proteins (Piscitelli et al. 2016).

3.2 Immobilization of the fusion protein Vmh2-GFP

Several tests of the immobilization of the fusion protein on multiwell plates were carried out using Vmh2-GFP at different concentrations (Table 1, rows S1÷S6), and evaluating the effect due to the presence of a preformed layer of native Vmh2 and due to a mixture of the fusion protein with the native Vmh2 (Table 1, rows S7 and S8).

TABLE 1. IMMOBILIZATION YIELD

The fusion protein was easily immobilized in the polystyrene well by deposition, resulting fluorescent and tightly bound to the surface after several washes with the buffer. A noticeably high immobilization yield of the fusion protein, in terms of both the fluorescence intensity and the protein amount, was calculated in all the tested conditions, in comparison with the opportune controls (Table 1, rows C1 and C2). In relation to the protein concentration used, there was a slight increase in the immobilization yield from 0.05 to 0.1 mg mL-1, while a further increase in this concentration did not affect this parameter. The immobilization yield of the fusion protein on a preformed Vmh2 layer was lower than that obtained by its direct deposition on the polystyrene surface (Table 1, row S7 and S2). As already reported (Piscitelli et al. 2016), the yield obtained through deposition on a preformed Vmh2 layer was higher for the fusion protein than for the GFP alone (Table 1, row S7 and C1). In the case of the immobilization of mixtures of the native Vmh2

and the Vmh2-GFP fusion protein, the measured immobilization yield was lower than that obtained for each protein separately (Table 1, row S8, S2 and C3) at the same final protein concentration. This result could be ascribed to their higher propensity for assembling in bulk solution rather than for coating when the two proteins are mixed. A similar effect has been observed for mixtures of the native Vmh2 and the Vmh2-GST fusion protein (Piscitelli et al. 2016).

The stability of the immobilized protein with respect to that of the free counterpart was also evaluated by measuring the fluorescence of Vmh2-GFP over time at two different temperatures (4°C and room temperature). The immobilized fusion protein Vmh2-GFP was fully stable for at least 30 days, while the free protein exhibited a complete loss of fluorescence after only three days at both temperatures. These results demonstrated that the stability of the GFP noticeably increased upon immobilization.

3.3 Thrombin assay

Considering that the surface active protein Vmh2 and the fluorescent GFP moieties were linked by the thrombin recognition site, Vmh2-GFP was used to specifically detect thrombin. The protease recognizes and cleaves its specific cutting site, releasing the GFP protein bound to the polystyrene surface through Vmh2 adhesion. As expected, in the presence of increasing concentrations of thrombin, the fluorescence emission intensity decreased due to the removal of GFP (Fig. 2).

FIGURE 2. SCHEME OF THE BIOSENSING STRATEGY.

Figure 3 shows the relationship between the percentages of fluorescence intensity measured after thrombin cleavage and the concentrations of thrombin. The results are reported in a logarithmic scale in order to highlight that fluorescence changed in a range of several orders of magnitude. When high Vmh2-GFP concentrations (0.1 and 0.2 mg mL^{-1}) were immobilized, a non-linear trend was observed (Fig. 3A, 3B). This phenomenon may be probably due to the existence of a non-homogeneous layer affecting the accessibility of the protease to its cleavage sites. As a fact, the

presence of fibrils observed at those Vmh2-GFP concentrations (Fig.1D) makes the film more irregular.

FIGURE 3. CALIBRATION CURVE.

To verify this hypothesis, lower protein concentrations (0.05 and 0.07 mg mL^{-1}) were immobilized on the 96-well plate. At the lowest concentration of Vmh2-GFP (0.05 mg mL^{-1}), a linear trend was observed in the whole concentration range of thrombin (Fig. 3D). Therefore, it may be inferred that the presence of the fibrils affected the homogeneity of the layer and, accordingly, the protease accessibility.

Actually, the procedure set-up at the lowest Vmh2-GFP concentration showed a very large linear range, from aM to mM. To the best of our knowledge this is the widest linear range obtained for a thrombin biosensor. This remarkable result may be due to the peculiar immobilization efficiency of our sensing molecule.

~~Next,~~ The relationship between the Vmh2-GFP fluorescence and the thrombin concentration was analysed by fitting the data using the linear equation (Equation 1):

$$y = a + b \log x \qquad (1)$$

The value of *a* resulting from the best fit procedure (Table S1) was compatible with the measured blank (111.5±0.6 a.u.) and it can be used to estimate the LOD. The LOD was calculated as 2.3 aM, according to the $3s_b/m$ criterion, where m is the slope of the calibration curve and s_b is the standard deviation of the blank (Table S1). ~~by subtracting three standard deviations from the intercept value *a*, and the corresponding value (108.4 a.u.) was considered reliable to detect the presence of thrombin. From Equation 1 the value of the thrombin concentration producing such a fluorescence intensity was 2.3 aM.~~ ~~To the best of our knowledge this is the lowest LOD~~ This value ~~found for thrombin detection,~~ is comparable to the lowest LOD found for thrombin detection, ~~that~~ obtained by Lu and co-workers (2015) (2.7 aM) using a bi-functionalized aptasensor.

The specificity of this assay was tested by evaluating the effect of other proteases on the fluorescence intensity (Fig. 4A). A decrease in the fluorescence intensity was measured only in the presence of thrombin, while no decrease was detected when other proteases were used. Moreover, the presence of high concentrations of other proteins (lysozyme, or bovine serum albumin) did not affect the decrease of fluorescence intensity due to the thrombin action (Fig. 4B).

FIGURE 4. ASSAY SPECIFICITY.

3.4 Thrombin assay in the plasma

Since a major challenge for thrombin detection is its quantification in complex biological fluids, the same assay was performed by adding different amounts of thrombin to healthy human plasma samples. A very similar response for thrombin in the buffer and in the plasma was observed (Fig. S2 and Table S1).

The GFP fluorescent biosensor was used to monitor the thrombin level in human plasma after the activation of its precursor prothrombin by human factor Xa (Table 2). Thrombin was also spiked in the plasma sample and its concentration was then evaluated in activated and non-activated samples (Table 2). The results obtained confirmed the reliability of this biosensor system in quantitative thrombin detection.

3. Conclusion

The first outcome of this work was the heterologous production in the yeast *P. pastoris* of the GFP protein endowed with robust adhesion ability thanks to its fusion to the class I hydrophobin Vmh2. In principle, the set-up system allows the straight immobilization of any protein fused to Vmh2 produced and secreted by *P. pastoris*.

The peculiar ability of Vmh2-GFP to adhere to surfaces allowed us to develop an alternative ~~In conclusion, this~~ method for the monitoring of thrombin in biological samples, ~~is a promising alternative approach,~~ compared to ~~the analytical methods~~ those currently in use~~, to detect thrombin in the plasma~~. The main advantages of this sensor include the simplicity of its design and

preparation, ~~and its~~ high sensitivity, wide range of linearity and accuracy. In fact, the Vmh2-GFP based biosensor allows a reliable detection of thrombin in the aM- mM range using a small amount of blood, and requiring a low consumption of reagents and time.

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Supplementary material. Figure S1. Characterization of Vmh2-GFP; **Figure S2.** Logarithmic plot of the calibration curve of the fluorescence intensity of the Vmh2-GFP *vs* the thrombin concentrations; **Table S1.** Parameters of linear fitting.

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Table 1. Immobilization yield of Vmh2-GFP

Conditions	Sample	Immobilization Yield (% mg tot)	Immobilization Yield (% Fluorescence Intensity)
S1	Vmh2-GFP 0.05 mg mL^{-1}	80	79
S2	Vmh2-GFP 0.1 mg mL^{-1}	95	97
S3	Vmh2-GFP 0.2 mg mL^{-1}	95	96
S4	Vmh2-GFP 0.5 mg mL^{-1}	95	96
S5	Vmh2-GFP 0.7 mg mL^{-1}	95	95
S6	Vmh2-GFP 1 mg mL^{-1}	95	95
S7	Vmh2-GFP on Vmh2 layer	75	78
S8	Vmh2-GFP: Vmh2 mix (1:3)	70	68
C1	GFP on Vmh2 layer	20	18
C2	GFP	7	6
C3	Vmh2	90	-

Table 2. *Assay results of thrombin in plasma*

Sample	Added amount (µM)	Expected amount (µM)	Measured amount (µM)
Plasma	--	--	< LOD
Plasma + factor Xa	--	--	$(9.89\pm0.06)\times10^{-1}$
Plasma	4.5×10^{-3}	4.5×10^{-3}	$(4.50\pm0.01)\times10^{-3}$
Plasma + factor Xa	4.5×10^{-3}	0.99	1.04 ± 0.01

Plasma	4.5	4.5	4.40±0.02
Plasma + factor Xa	4.5	5.49	5.52±0.04

Highlights

- A self-immobilizing protein formed by a hydrophobin and a Green Fluorescent Protein was produced
- The obtained amyloid-like fibrils were able to adhere to polystyrene surface
- Vmh2-GFP fusion protein has proven to be a smart and effective tool for the study of Vmh2 self-assembling
- An ultra-sensitive biosensor for detection of thrombin in plasma was developed

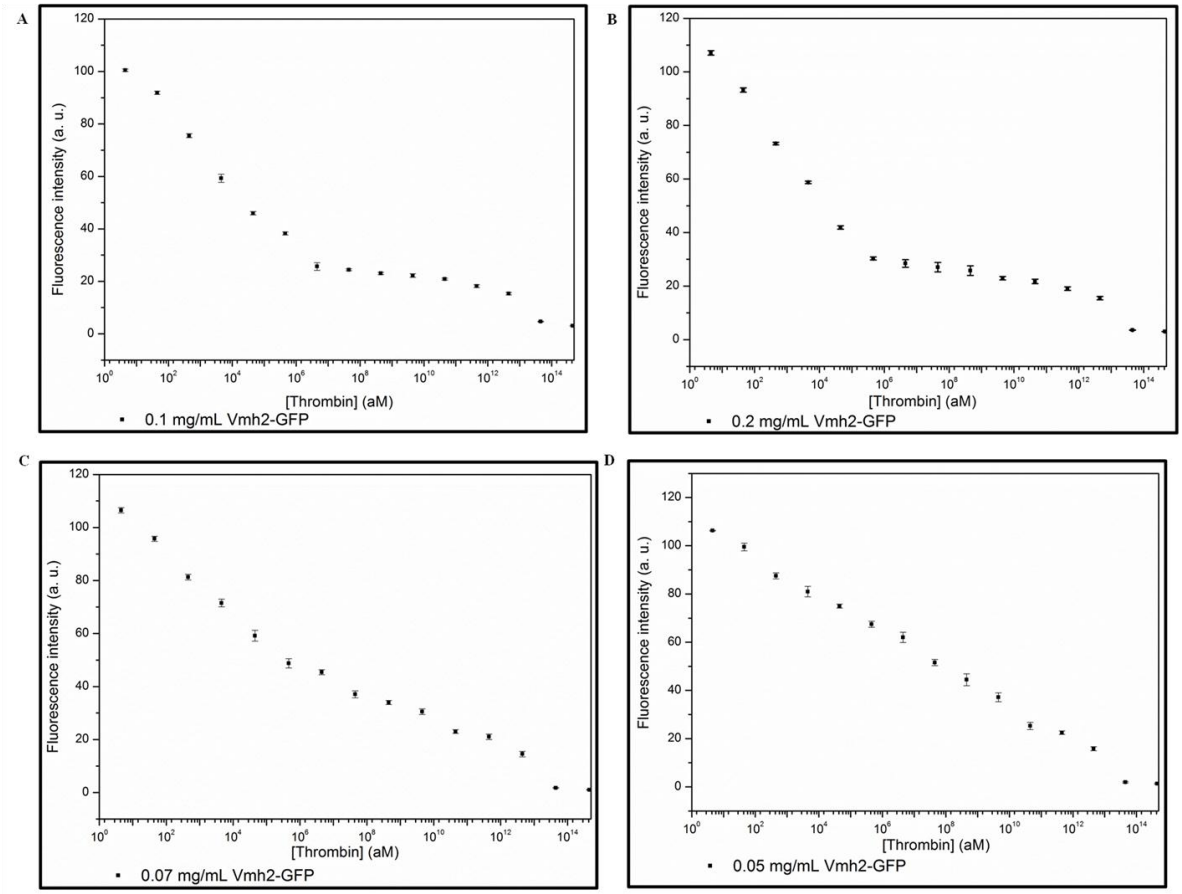
FIGURE LEGENDS

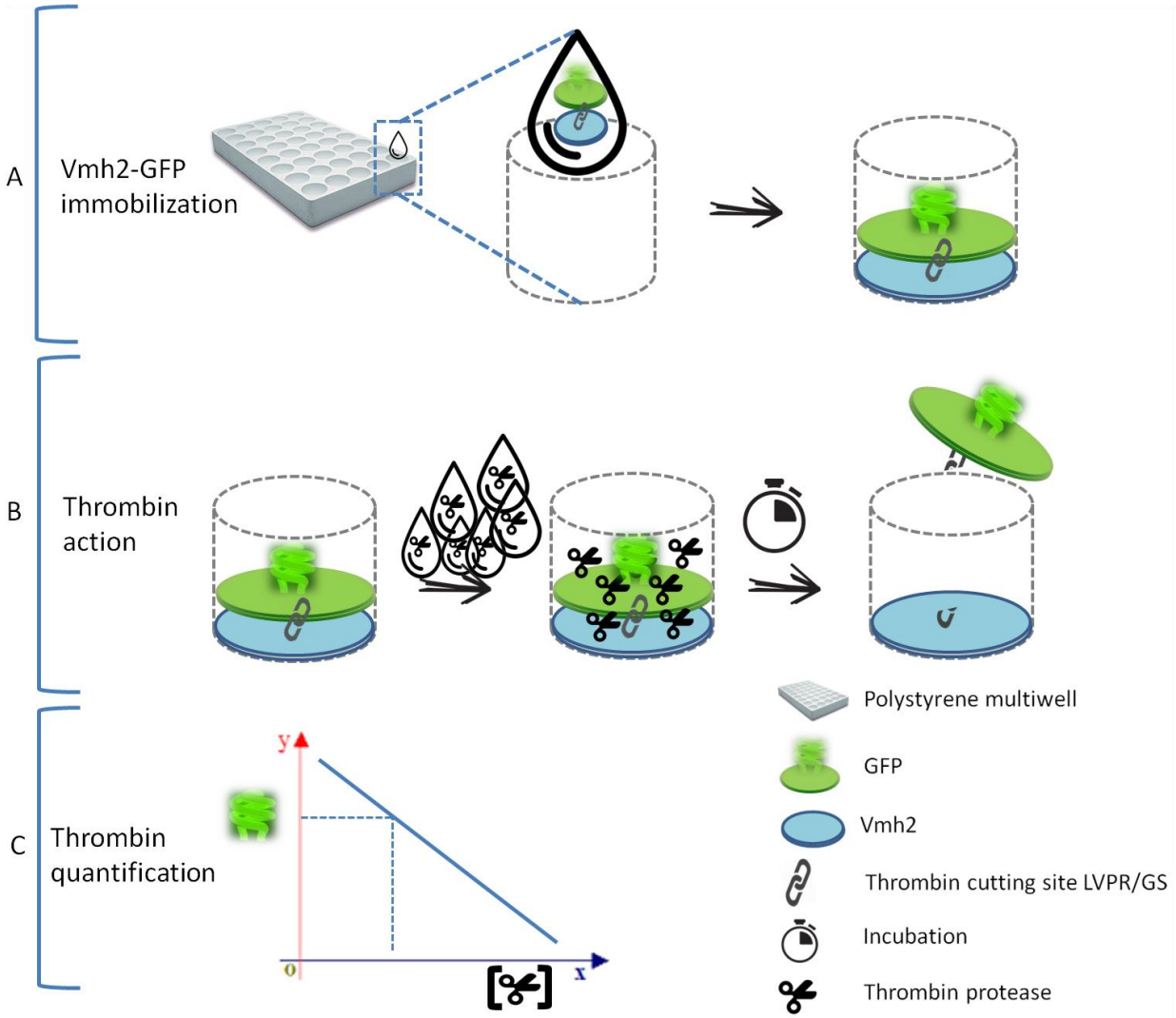
Figure 1. Protein characterization. A. Fluorescence images of a bubble dispersion of VMh2-GFP. **B.** Measurement of water contact angle of a water drop on the polystyrene surface after the deposition of GFP, Vmh2 and Vmh2-GFP. The polystyrene surface was used as a reference. **C.** Fluorescence spectra of 30 μM ThT after the immobilization of different Vmh2-GFP concentrations. **D**.Immobilization of different Vmh2-GFP concentrations on the polystyrene surface analysed by confocal microscopy.

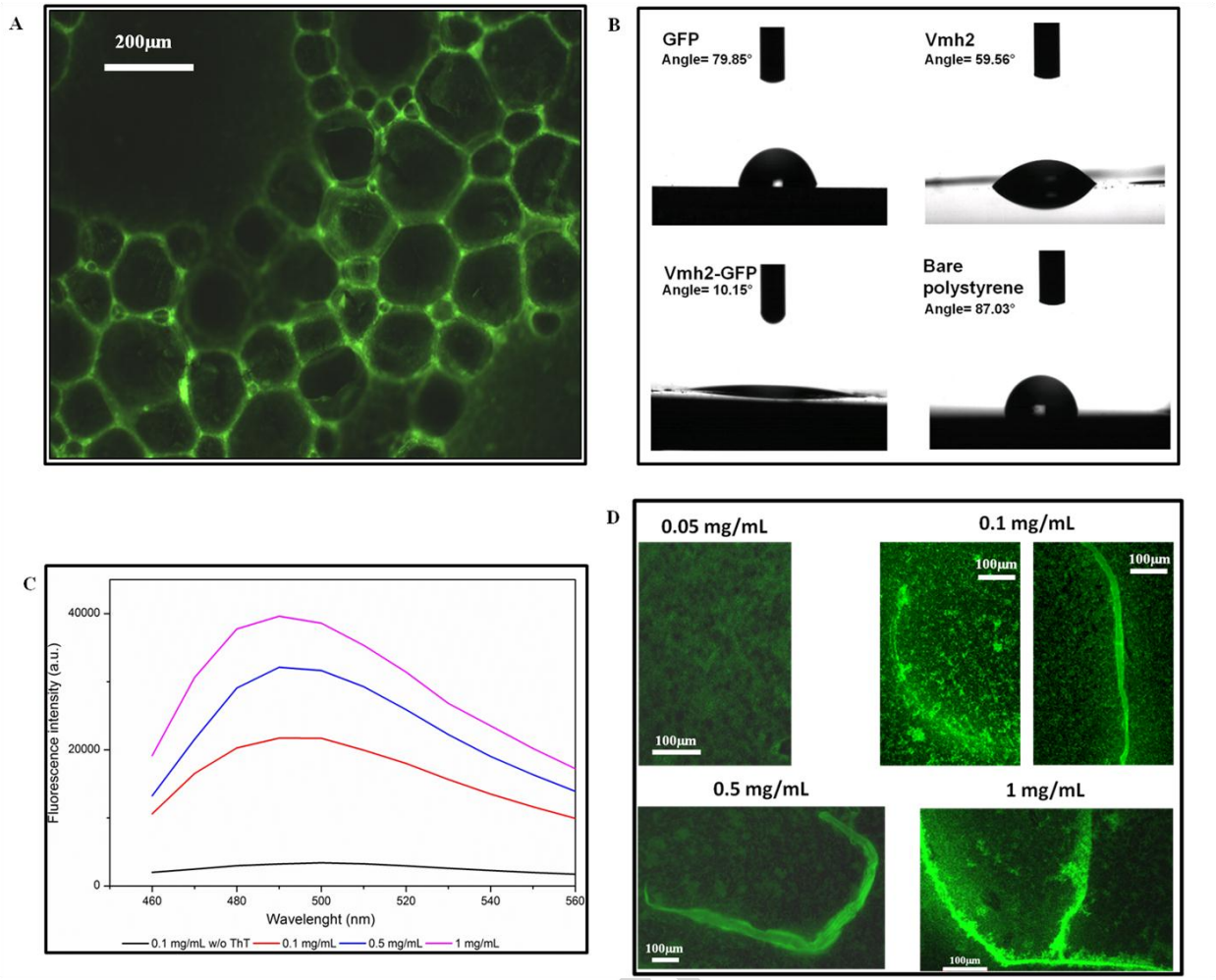
Figure 2. Scheme of biosensor. Schematic representation of the sensing procedure for detection of thrombin.

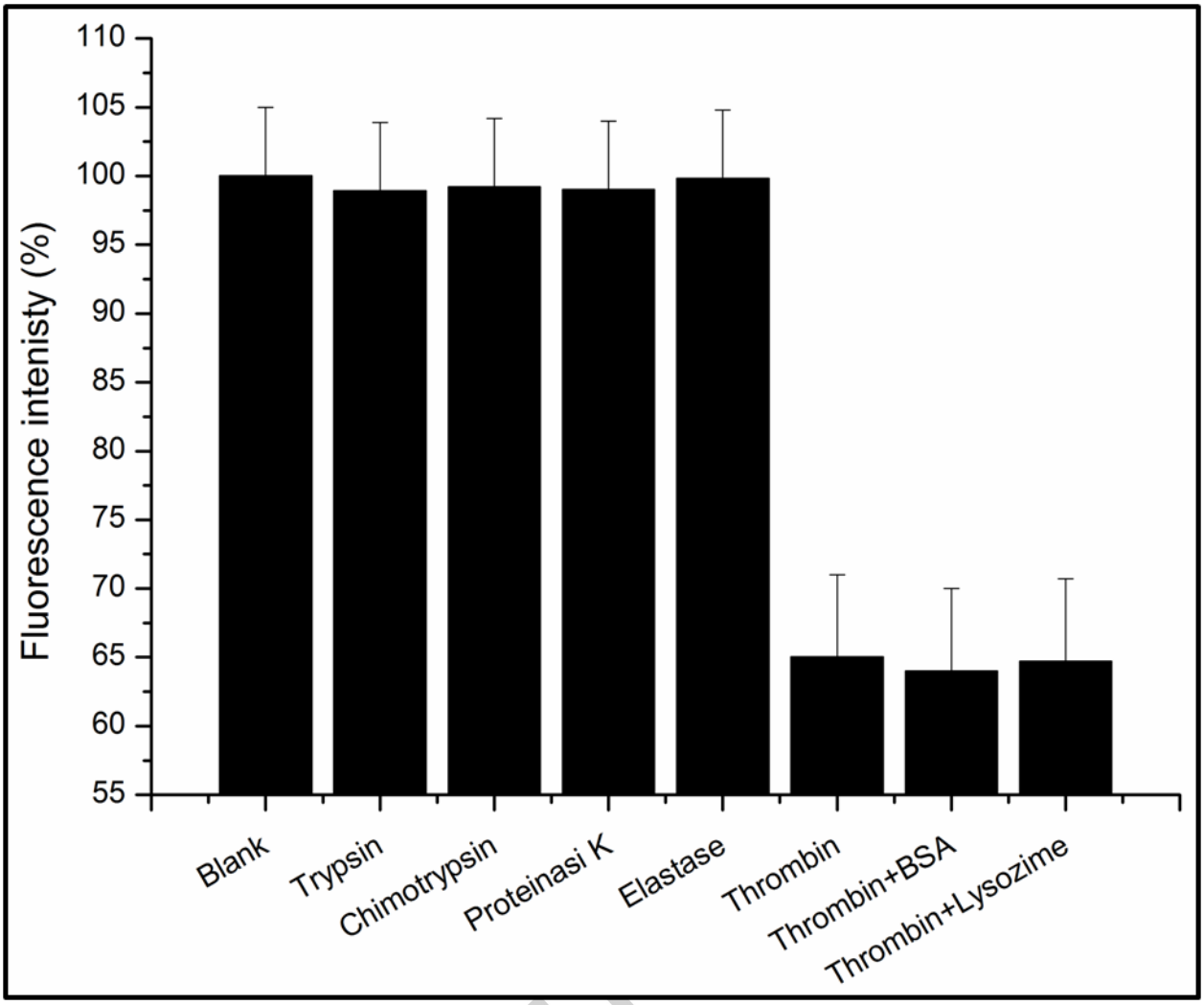
Figure 3. Calibration curve. Logarithmic plot of the calibration curve of the fluorescence intensity emission (top reading mode) of Vmh2-GFP at 505 nm *vs* the thrombin concentration in Tris-HCl buffer at pH 8.0. The Vmh2-GFP immobilization was performed starting from different protein concentrations. **A.** 0.1 mg mL^{-1}. **B.** 0.2 mg mL^{-1}. **C.** 0.07 mg mL^{-1}. **D.** 0.05 mg mL^{-1}. Coefficients of variation (CV%, n=5) vary from 0.2 to 8.2%.

Figure 4. Specificity of the assay. Percentage (%) of the fluorescence intensity of Vmh2-GFP in the presence of ~~**A.** 4.5 μM~~ ftM of different proteases, such as thrombin, trypsin, chimotrypsin, proteinase K, elastase ,and of 4.5 ftM of thrombin with 3.0 μM lysozyme or BSA. ~~**B.** 3.1 μM lisozyme, 1.5 μM BSA~~.




A
Vmh2-GFP
immobilization
B
Thrombin
action
C
Thrombin
quantification
y
x
0
[✂]
Polystyrene multiwell
GFP
Vmh2
Thrombin cutting site LVPR/GS
Incubation
Thrombin protease


A
200µm
B
GFP
Angle= 79.85°
Vmh2
Angle= 59.56°
Vmh2-GFP
Angle= 10.15°
Bare
polystyrene
Angle= 87.03°
C
Fluorescence intensity (a.u.)
40000
20000
0
460
480
500
520
540
560
Wavelenght (nm)
0.1 mg/mL w/o ThT
0.1 mg/mL
0.5 mg/mL
1 mg/mL
D
0.05 mg/mL
0.1 mg/mL
0.5 mg/mL
1 mg/mL
100µm


Fluorescence intenisty (%)
110
105
100
95
90
85
80
75
70
65
60
55
Blank
Trypsin
Chimotrypsin
Proteinasi K
Elastase
Thrombin
Thrombin+BSA
Thrombin+Lysozime