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# Aptamer-functionalized solid phase microextraction–liquid chromatography/tandem mass spectrometry for selective enrichment and determination of thrombin☆

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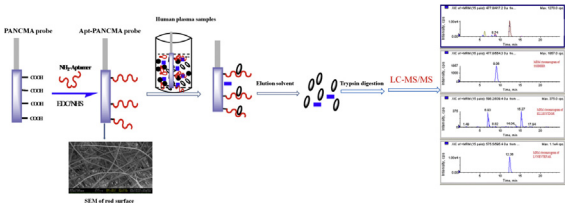
## HIGHLIGHTS

- DNA-aptamer functionalized polymer coating was prepared.
- The electrospun polymeric coating has porous and fibrous structure.
- The selective coating was used for solid phase microextraction of thrombin.
- The method is sensitive, reusable and has potential to use *in vivo*.

## GRAPHICAL ABSTRACT

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## ABSTRACT

In this publication, a novel solid phase microextraction (SPME) coating functionalized with a DNA aptamer for selective enrichment of a low abundance protein from diluted human plasma is described. This approach is based on the covalent immobilization of an aptamer ligand on electrospun microfibers made with the hydrophilic polymer poly(acrylonitrile-co-maleic acid) (PANCMA) on stainless steel rods. A plasma protein, human α-thrombin, was employed as a model protein for selective extraction by the developed Apt-SPME probe, and the detection was carried out with liquid chromatography/tandem mass spectrometry (LC–MS/MS). The SPME probe exhibited highly selective capture, good binding capacity, high stability and good repeatability for the extraction of thrombin. The protein selective probe was employed for direct extraction of thrombin from 20-fold diluted human plasma samples without any other purification. The Apt-SPME method coupled with LC–MS/MS provided a good linear dynamic range of 0.5–50 nM in diluted human plasma with a good correlation coefficient ($R^2$ = 0.9923), and the detection limit of the proposed method was found to be 0.30 nM. Finally, the Apt-SPME coupled with LC–MS/MS method was successfully utilized for the determination of thrombin in clinical human plasma samples. One shortcoming of the method is its reduced efficiency in undiluted human plasma compared to the standard solution. Nevertheless, this new aptamer affinity-based SPME probe opens up the possibility of selective enrichment of a given targeted protein from complex sample either *in vivo* or *ex vivo*.



## 1. Introduction

Solid phase microextraction (SPME) is a simple, environment friendly sample preparation technique first introduced in 1990 [1]. It offers many advantages over conventional sample preparation methods by integrating sampling, extraction, concentration, and sample introduction into a single step, while minimizing or

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completely eliminating the use of solvents. SPME has been successfully applied in many sectors within the field of analytical chemistry, including environmental analysis [2], food and fragrance analysis [3], bioanalysis [4,5], drug analysis [6], and metabolomics studies [7,8]. The coupling of SPME with LC–MS/MS has successfully been implemented for both *ex vivo* and *in vivo* analysis of drugs in biological matrices [9–11]. The development of novel coatings has allowed for improved throughput, biocompatibility and robustness of the SPME LC–MS/MS methods for various target analytes. However, based on their partition coefficients, most coatings can extract a class of analytes that leads to quantification complications; this is often due to the co-extraction of undesired species or displacement by stronger adsorbents present in complex biological matrices such as blood, plasma and serum. It has been proposed that improvements in coating selectivity can potentially circumvent the challenges of competition, displacement and non-specific binding. Inspired by the traditional immunoassay technologies, the immobilization of antibodies on SPME fibers has been applied successfully to extract drug molecules from human serum samples [12–14]. While antibody-based coatings have shown very good selectivity for the analytes in serum samples, the limited capacity of such coatings restricts quantifications in very low dynamic ranges.

For the development of selective and biocompatible SPME coatings, aptamers are a valid alternative to antibodies or other receptors due to the numerous unprecedented advantages of aptamers such as high specificity and affinity, high reproducibility, superior stability, versatile target binding, and low cost of development [15–17]. These unique properties make aptamers perfectly suitable for biosensing, diagnostics, therapeutics, and separation sciences [9,17–20]. To date, many aptamer-based affinity approaches, including aptamer-based chromatographies, aptamer-based capillary electrophoresis, and aptamer-based microfluidics, have been successfully developed for extraction, separation, purification, and detection of targets of interest, ranging from small molecules to proteins and cells [16,19]. During the preparation of this manuscript, the application of aptamers for selective extraction of adenosine from human plasma was encountered as well [21]. The authors have successfully demonstrated the benefits of using aptamers over other traditional coatings for detection of small polar analytes. Therefore, an aptamer-based SPME method is very promising for selective analysis of targets of interest in biofluids.

So far, most of the developed SPME methods to date have been focused on small target analytes [5,7]. Macromolecules, such as proteins in biofluids, are attractive targets for biomarker or drug discovery. In spite of the advances in mass spectrometry, the quantification of low-abundance proteins in plasma and serum remains a challenge due to the level of sample heterogeneity along with the technical robustness and throughput required for routine clinical assays. Recently, mass spectrometric immunoassay (MSIA)-based targeted protein assays have emerged as an attractive alternative to traditional immunoassay methods such as enzyme-linked immunosorbent assays (ELISA) [22,23]. Moreover, numerous protein-enrichment approaches for complexity reduction in plasma and serum have been developed to detect trace level proteins, including pipette tip based solid phase extraction (SPE), nanoparticles or immunoaffinity based magnetic beads [24–26]. Besides the wide application of the pipette tip format, they are prone to be clogged with viscous biological samples during the repeated aspirations leading to inefficient sample enrichment. We envisaged that the open-bed format of SPME probe will provide efficient protein enrichment by providing no clogging and reduced sample interference. To demonstrate the applicability of SPME for enriching low-abundance proteins from human plasma, we have chosen thrombin as a model protein. Thrombin is a specific serine protease that plays multifunctional roles in blood coagulation cascade, thrombosis and haemostasis. It acts as a key arbiter to regulate the balance between the procoagulant and anticoagulant pathways by virtue of its dual role [27]. As such, the use of human thrombin has been approved by the US Food and Drug Administration to help control bleeding during surgeries [28]. Due to its biological significance and practical applications in medicine, it is important and necessary to develop analytical methods capable of high sensitivity in the detection of thrombin in blood. Such methods could be used to assist in the cure of patients suffering from diseases known to be associated with coagulation abnormalities, as well as to evaluate the effectiveness of anticoagulant therapy [29]. At present, several analytical methods, especially aptamer-based methods, have been developed for determination of thrombin [30–33]. In past research, it has been demonstrated that the anti-thrombin DNA aptamer exhibits good specific recognition and selective enrichment of thrombin in biological samples when the aptamer was immobilized onto magnetic beads [31], organic–inorganic hybrid silica [33], gold nanoparticles [34], poly(acrylic acid) functionalized upconverting phosphors [35], grapheme [36], or poly(pyrrole-nitrilotriacetic acid) [37].

The aim of this study was to develop an aptamer-based novel selective SPME probe for human α-thrombin in plasma samples with the aid of liquid chromatography/tandem mass spectrometry (LC–MS/MS). At first, a carboxy-functionalized microfiber structure was prepared by electrospinning a poly(acrylonitrile-co-maleic acid) (PANCMA) co-polymer on pre-cleaned stainless steel rods. The 29-mer DNA aptamer selective to the heparin binding site of the thrombin was then immobilized on the polymer substrate, providing the aptamer-based SPME (Apt-SPME) probe. Prepared Apt-PANCMA probes were then evaluated in terms of selectivity, binding capacity, extraction ability and reusability. Under optimal conditions, the proposed Apt-SPME coupled with LC–MS/MS has been successfully applied to the analysis of thrombin in real human plasma samples.

## 2. Experimental

### 2.1. Chemicals and materials

Human α-thrombin and prothrombin were purchased from Haematologic Technologies Inc. Human serum albumin, human hemoglobin, cytochrome C, trypsin, maleic anhydride (≥99.0%), acrylonitrile (≥99.0%), formic acid (98%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, ≥99.0%), *N*-hydroxysuccinimide (NHS, 98%), potassium persulfate (≥99.0%), and anhydrous sodium sulfite (≥98.0%) were purchased from Sigma–Aldrich (St. Louis, MO). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Unionville, Ontario, Canada). *N*,*N*-dimethylformamide (DMF) was obtained from Caledon Labs (Ontario, Canada). An anti-thrombin DNA aptamer (Apt) with an amine terminal group (5′/5AmMC6/-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′) was purchased from Integrated DNA Technologies Inc. (Canada). The internal standard peptide was purchased from AnaSpec Incorporation (Fremont, CA, USA), and the amino acid sequence is SSIIHIER. Clinical human plasma from a patient was kindly donated by Toronto Hospital; other human plasma samples were purchased from Lampire Biological Laboratories (LBL), Inc. (Pipersville, PA).

All solutions were prepared with ultrapure water purified by a Barnstead/Thermodyne NANO pure water system (Dubuque, IA, USA). Solutions containing 1.0 mg $mL^{-1}$ of thrombin were prepared in a phosphate buffered saline (PBS) buffer solution (10 mM, pH 7.4) and a $NH_4HCO_3$ buffer (25 mM), respectively. The two stock solutions were stored at 5 °C in a refrigerator.

### 2.2. Preparation of poly(acrylonitrile-co-maleic acid)

Poly(acrylonitrile-co-maleic acid) (PANCMA) was prepared by using a free radical water-phase precipitation polymerization according to the method described by Nie et al. with minor modifications [38]. Briefly, 7.4 g maleic anhydride, 10.6 g acrylonitrile and 20 mL of deionized water at 60 °C were added into a reactor equipped with a mechanical stirrer, thermometer, and nitrogen inlet tube. Then, 135 mg $K_2S_2O_8$ and 75 mg $Na_2SO_3$ were added into the stirring solution while maintaining the reaction temperature at 60 °C under nitrogen atmosphere. The pH value of the mixture was adjusted to around 2 using a dilute $H_2SO_4$ solution. The copolymerization was continued for 3 h, and the precipitated copolymer was filtered and washed with excess de-ionized water and ethanol to remove residual monomers. The obtained PANCMA was dried for 12 h under vacuum at 60 °C.

### 2.3. Preparation of the PANCMA probe via electrospinning

The electrospinning process was similar to the method described by Xu et al. [39,40]. For this procedure, 4.0 g PANCMA was dissolved in 50 mL of DMF at room temperature with gentle stirring for 12 h. The obtained homogeneous solution was placed in a syringe bearing a 1.0 mm inner diameter metal needle connected to a high voltage power supply (UW-SYS E2047, University of Waterloo, Canada). A grounded counter electrode was connected to the stainless steel rod-collector (55 mm × 1.5 mm, i.d.), which was rapidly rotated by an electrical power unit during electrospinning experiments. According to experimental results, the optimal electrospinning voltage was 10.0 kV, the distance between the needle tip and the collector was 130 mm, the flow rate of the solution (controlled by a Kd Scientific syringe pump (Holliston, MA, USA)) was 0.30 mL $h^{-1}$, the coating length of probe-collector was 30 mm, and the collection time was 50 min. The obtained membrane on the probe was dried for at least 3 h at 60 °C in vacuum oven before it was used.

### 2.4. Aptamer immobilizing on the surface of the PANCMA

As shown in Scheme 1, for aptamer immobilization, the PANCMA matrix was first activated with EDC/NHS dissolved in 50 mM PBS buffer (pH 8.0), and then the amine functionalized DNA aptamer was added for preparation of Apt-PANCMA. Briefly, four PANCMA probes were thoroughly washed with water and rinsed with a 50 mM PBS solution containing 20 mM KCl and 600 mM NaCl (pH 8.0). After this, the pretreated probes were submerged into 2.0 mL of EDC/NHS solution (100 mM EDC and 100 mM NHS in 50 mM PBS buffer, pH 8.0) and shaken gently at room temperature to activate the —COOH group of PANCMA. After incubation for 2 h, 240 nmol aptamer was added and incubated overnight. The prepared aptamer functionalized PANCMA (Apt-PANCMA) probes were taken out and washed several times with a 10 mM PBS buffer (pH 7.4), then dipped into the same PBS solution for future use.

The microstructure of the prepared Apt-SPME probe was investigated with a scanning electron microscope (SEM, Zeiss ULTRA plus).

The aptamer concentration in sample solution before and after the immobilizing reaction was measured by a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific).

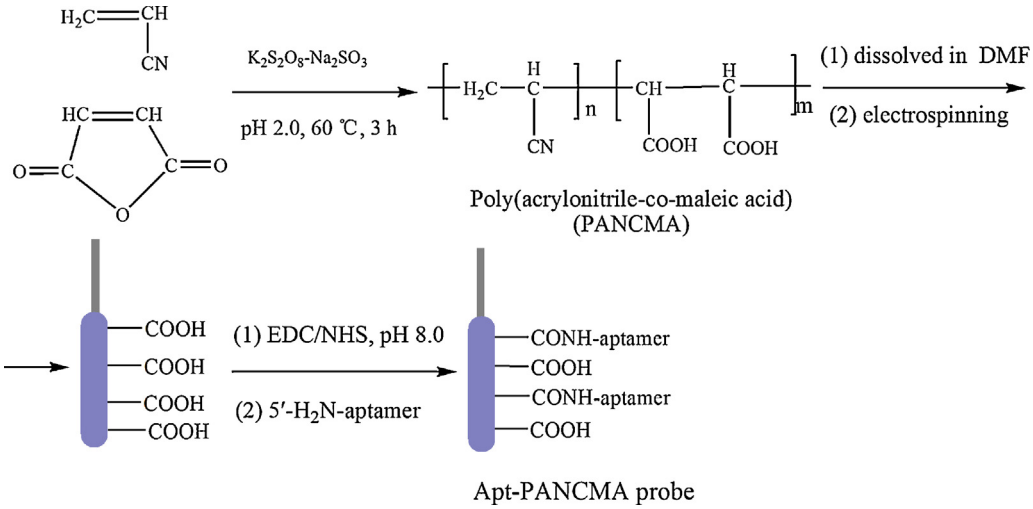
### 2.5. Thrombin capture on Apt-SPME probes

Briefly, Apt-SPME probes were first washed with 10 mL of PBS (10 mM, pH 7.4), then dipped into 2.0 mL of sample solution. After incubation for 1.0 h at room temperature under shaking (130 rpm, SK-300 SHAKER, JEIO TECH, Ontario, Canada), the probe was taken out from the sample solution, washed three times with 2 mL of PBS, then consecutively washed 3 times with 10 mL of pure water to remove any unspecific bound or weakly bound species. The target analyte specifically bound on the Apt-SPME probe was eluted with 2 mL of eluting solution (acetonitrile/water, 80/20, v/v). The elution solution was collected and then dried at ambient temperature with $N_2$ gas. Finally, the probes were regenerated by successive washes with 10 mL of PBS, and stored in the PBS buffer solution at 5 °C.

The obtained residual sample was dissolved in 146 uL $NH_4HCO_3$ buffer (25 mM), and then 30 μL of trypsin solution (1.0 mg $mL^{-1}$ in $NH_4HCO_3$ buffer) was added. The obtained sample solutions were incubated overnight at 37 °C. After that, a 4 μL internal peptide SSIIHIER solution (500 nM in formic acid/acetonitrile/water (0.1/10/90, v/v/v) solution) and 20 μL acetonitrile/formic acid (80/20, v/v) were added into the digestion solution, then filtered by a 0.22 μm Supor® membrane (Pall Corporation, USA) for LC–MS/MS analysis.

### 2.6. Selectivity and binding capacity on Apt-SPME probe

To demonstrate the selectivity on the Apt-PANCMA probe for thrombin, prothrombin, hemoglobin, human serum albumin, and




**Scheme 1.** Schematic representation of the processes for preparation of aptamer functionalized SPME probe (Apt-SPME) probe.

cytochrome C were chosen as reference proteins. The similar extraction procedure was described briefly in Supporting Information.

The binding capacity of thrombin on a single Apt-PANCMA probe was evaluated by determining the recovery amount of thrombin with the increase of the concentration of standard thrombin in PBS buffer (10 mM, pH 7.4).

### 2.7. Recovery test and determination of thrombin in human plasma

To test the applicability of the proposed method to complex biological samples, we used 20-fold diluted human plasma as a sample matrix and determined the corresponding recovery of the spiked thrombin. Briefly, before extraction with the Apt-PANCMA probe, different amounts of thrombin were spiked into 20-fold diluted human plasma sample; the diluted human plasma samples spiked with thrombin (0, 0.5, 5, 10, 20, 50, 100, 120 and 150 nM) were then extracted and analyzed by the same procedure described above for thrombin capture and detection, respectively.

For analysis of real human plasma, the concentration of thrombin in human plasma was determined by using the calibration equation, which was obtained by measuring the intensity of the signal peptide from thrombin in relation to the amount of spiked thrombin, ranging from 0.5 to 150 nM in 20-fold diluted human plasma.

### 2.8. Instrumentation and operating conditions

A Shimadzu (LC-10AD) high pressure liquid chromatography (HPLC) system (Kyoto, Japan) coupled to an API 4000 mass spectrometer (AB Sciex, Concord, Ontario, Canada) equipped with a TurboIonSpray source was used in the qualitative and quantitative analysis of thrombin. Instrument control was performed using the Analyst 1.5 software. A CTC PAL autosampler platform from Leap Technologies (CTC Analytics, NC, U.S.) was used to inject 20 μL of samples for LC–MS/MS analysis. The LC separations were performed on a BioBasic-C8 column (100 mm × 1.0 mm i.d., particle size 5 μm) from Thermo Scientific (Waltham, MA, USA). The sample oven temperature was maintained at 5 °C, and the column was at ambient temperature. The mobile phases consisted of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B), and the flow rate was 120 μL $min^{-1}$. According to our preliminary experiments for thrombin analysis, the optimal gradient profile was as follows (min/% of mobile phase B): 0.0/5, 0.5/5, 20.5/25, 21.0/99, 26.0/99, 27.0/5, and 47.0/5.

The MS/MS analysis in API 4000 mass spectrometer was performed in positive mode under multiple reaction monitoring (MRM) conditions at 477.76 → 417.188, 477.76 → 554.31 and 477.76 → 667.452 for internal peptide SSIIHIER, and 598.20 → 460.50, 598.20 → 623.70, 598.20 → 710.80 and 598.20 → 839.38 for ELLESYIDGR, respectively, and typical MS/MS spectra of $[M+2H]^{2+}$ of ELLESYIDGR are shown in Fig. S-2 in SupportingInformation. According to our preliminary experiments for determination of thrombin, the ionspray voltage and source temperature were set at 5000 V and 400 °C, and collision gas, curtain gas, ion source gas 1, and ion source gas 2 were optimized at 4, 10, 20 and 0 (arbitrary units), respectively. The optimized value for declustering potential (DP), entrance potential (EP), collision-induced dissociation energies (CE), and collision cell exit potential (CXP) were 20, 10, 30, and 15 V, respectively. For the other three proteins, the same MRM conditions were used; specific signal peptides used for determination of each protein are shown in Table S-1 in Supporting Information.

### 2.9. Method validation

After acquisition, the specific ions were extracted from the spectra using the Analyst 1.5 software. Identification was based on retention time, accurate mass and product ions of the parent ions relative to external standards. The peak area of the corresponding extracted ion chromatogram (XIC) at 477.76 → 554.31 for internal peptide SSIIHIER and at 598.20 → 839.38 for ELLESYIDGR, respectively, was used to quantify the amount of thrombin based on the calibration curves. Method validation involved the determination of dynamic range, limit of detection (LOD, S/N = 3), accuracy, and precision according to the accepted criteria.

The calibration curve for LC–MS/MS analysis was built using a serial digestion solution of different thrombin concentrations. First, 200 nM thrombin in $NH_4HCO_3$ buffer (25 mM) was diluted to the desired concentration (1, 5, 10, 20, 50, 80, and 100 nM) with $NH_4HCO_3$ buffer, and 30 μL of trypsin solution (1.0 mg/mL in $NH_4HCO_3$ buffer) was added in the corresponding sample solution (final volume: 960 μL), respectively. The prepared sample solutions were incubated overnight at 37 °C. After that, 20 μL of 500 nM internal peptide SSIIHIER and 20 μL acetonitrile/formic acid (80/20, v/v) were added into the obtained digestion solution, respectively, and then the all samples were filtered by 0.22 μm Supor® membrane and analyzed by LC–MS/MS system. For the analysis of the other four proteins, a similar procedure was used. The obtained results are shown in Fig. S-1 and Table S-2 in Supporting Information.

Additionally, the accuracy of the proposed method was estimated by determining the recovery of thrombin at different concentration levels. Precision was confirmed by evaluating relative standard deviations (RSDs) of the retention time and the peak area of extract ion chromatogram (EIC). The limits of detection were calculated using a signal-to-noise ratio of 3 (S/N = 3, the ratio between the EIC peak intensity and the noise).

All data presented in this work was obtained by averaging a minimum of three replicates unless otherwise noted.

## 3. Results and discussion

### 3.1. Preparation and characterization of Apt-PANCMA probe

Efficient extraction of targeted protein analytes with an immobilization affinity ligand is highly dependent on the physicochemical properties of the solid support. The immobilization strategy of the aptamer onto a steel rod coated with a functional polymer is presented in Scheme 1. A polymeric support was chosen based on a combination of polyacrylonitrile (PAN) and maleic acid (MA). PAN provided porosity and physical strength, while the presence of MA was proven to improve hydrophilicity and chemical functionality [39]. Electrospinning was employed to create micro/nanofibers of the polymer as an inexpensive and simple method of obtaining a high surface area substrate [40]. Using this method, the polymer mat was attached to a stainless rod without the need of a binder. The fibrous structures possessing reactive carboxyl groups were used to immobilize DNA aptamers. In order to obtain maximum carboxyl groups without sacrificing the mechanical strength of the support, the ratio of acrylonitrile to maleic acid was increased from 2:1 to 8:1. Results showed that the prepared PANCMA was partly dissolved in water when the ratio was 2:1. With a ratio higher than 4:1, the polymer was chemically and mechanically stable; therefore, an optimal ratio of 4:1 was chosen for preparing the PANCMA support. Under the optimal electrospinning experimental conditions discussed in Section 2, the thickness of the obtained electrospun PANCMA fiber coating on the stainless steel rod was estimated to be approximately 60–65 μm. The thrombin-specific DNA aptamer modified with a 5′-amine functional group was covalently conjugated to the electrospun PANCMA support through the reactive carboxyl moieties *via* EDC/NHS protocol. The aptamer coupling efficiency was approximately 84.6%, evaluated by comparing the immobilized

aptamer amount on the SPME probe with the original quantity of aptamer added in the coupling reaction solution. Fig. 1(A) shows a uniform surface in terms of thickness throughout the Apt-PANCMA probe. Moreover, the surface morphology of the electrospun PANCMA fiber was not changed before or after reaction with the aptamer. Additionally, the SEM images (Fig. 1A and B) show that the diameters of the prepared fibers were about 1.0 μm, and that fibers possessed a highly porous surface, which should significantly increase the surface area availability on the probes. The spaces among the fibers are in the range of 200–5000 nm, which should facilitate faster protein mass transfer, leading to efficient analyte binding.

### 3.2. Specificity of Apt-SPME probes for thrombin

First of all, the specific capture ability of the Apt-SPME probe was evaluated by investigating the recovery of human α-thrombin, with the addition of prothrombin, hemoglobin, cytochrome C and human serum albumin (HSA) in PBS buffer. The obtained results shown in Fig. 2 indicated that the recovery of thrombin was significantly higher than that of the other proteins, which suggested that the prepared Apt-SPME probe was able to capture thrombin with high selectivity due to the specific interaction of thrombin with its aptamer on the Apt-SPME probe. In order to test the selectivity of the Apt-SPME probe for thrombin compared to its parent protein, prothrombin, a separate study was carried out. As shown in Fig. S-3, the percent recovery of prothrombin was less than 2% even with higher concentrations of prothrombin being spiked onto the samples. These results suggested that the immobilized aptamer is functional for selective extraction of thrombin. In order to test if the polymeric substrate extracts the target thrombin, the electrospun PANCMA substrate (without aptamer functionalized probe) was employed as a negative control to extract thrombin according to the same procedure. The results showed that the thrombin recovery on the control PANCMA-probe was 14.9%, which is significantly lower than the 87.8% obtained by using Apt-SPME probes, resulting from the high specific binding affinity of the 29-mer aptamer instead of the unspecific adsorption affinity of the matrix. Moreover, the lower non-specific extraction might be due to the high surface area of the electrospun microfiber-based SPME probe and the hydrophilic nature of the polymeric substrate used for aptamer immobilization.

### 3.3. Optimization of extraction, desorption and detection conditions

Extraction time is a fundamental parameter governing the efficiency of the extraction, and may be shortened by intensive stirring for the direct extraction mode [41]. In this work, the effect of extraction time was investigated by varying it from 10 to 120 min under moderate shaking (130 rpm). Fig. 3 shows that the extraction recovery of thrombin in PBS buffer increased as extraction time lapsed from 10 to 60 min, but was not obviously affected after 60 min extraction. Therefore, 60 min of extraction time was selected for the following experiments in order to effectively extract thrombin from samples.

To avoid a carry-over effect and to enhance sensitivity, rapid and effective desorption is necessary. Several desorption solvents (70% (v/v) methanol, 70% (v/v) acetonitrile, and 80% (v/v) acetonitrile) were tested to achieve a complete desorption of thrombin from Apt-SPME probes, and results showed that 2.0 mL of 80% (v/v) acetonitrile was found to be optimum for eluting thrombin from the probes.

In order to identify and determine thrombin in real samples, an Apt-PANCMA probe combined with LC–MS/MS has been used in this work. Thrombin was identified by the corresponding extracted ion chromatogram (XIC), retention time and mass spectrum of ELLESYIDGR, which is the strongest signal peptide from digestion

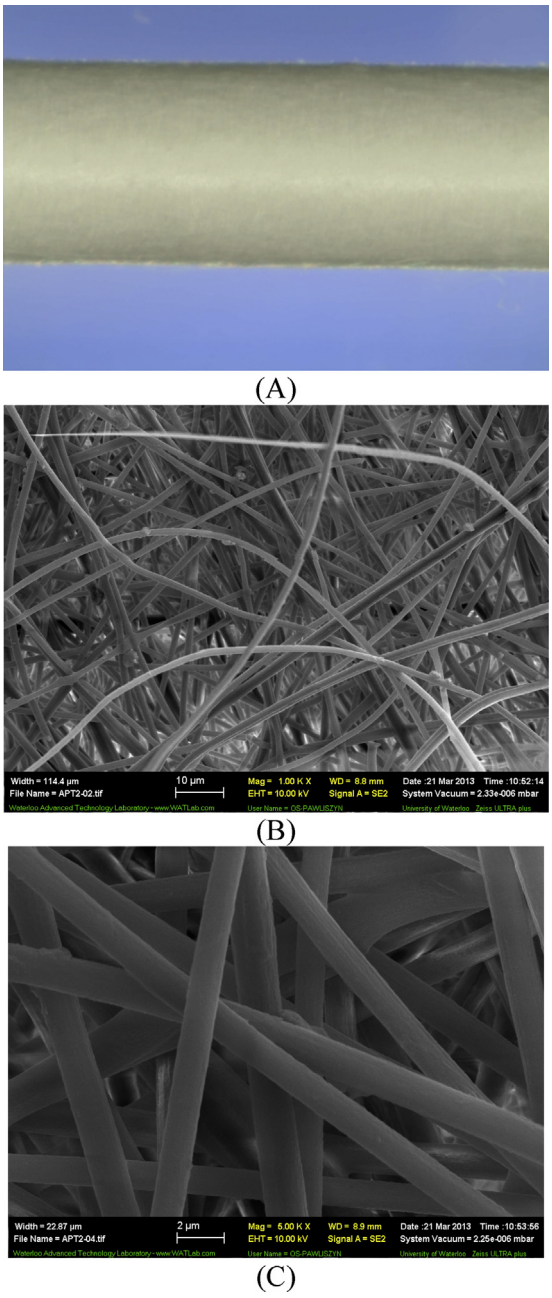



**Fig. 1.** Photographic image (A, 10×, 1.5 mm diameter) and SEM images of Apt-SPME probe (B, 1000×; C, 5000× magnification).

of thrombin in accordance with the results of Zhang et al. [42]. The obtained informative mass product, especially predominant y and b ions, can be found under optimal MRM conditions in Fig. S-2, available in Supporting Information.

The calibration curve for LC–MS/MS analysis was constructed by measuring the relative XIC intensity of the signal peptide ELLESYIDGR at $m/z$ 598.20 → 710.80 from thrombin and the internal standard peptide SSIIHIER at $m/z$ 477.76 → 554.31. Table S-2 shows that the calibration curves were linear over the concentration range of 1.0–100 nM with a good correlation coefficient ($R^2 = 0.9993$). The linear regression equation is $Y = 1.497 \times 10^{-2}\ X - 5.880 \times 10^{-3}$, where $Y$ stands for the logarithm of intensity ratio of the peak area of the selected signal peptide at $m/z$ 839.38 versus that of the internal peptide at $m/z$ 554.31, $X$ stands for the logarithm of concentration of thrombin in nanomolar. The detection limit of the

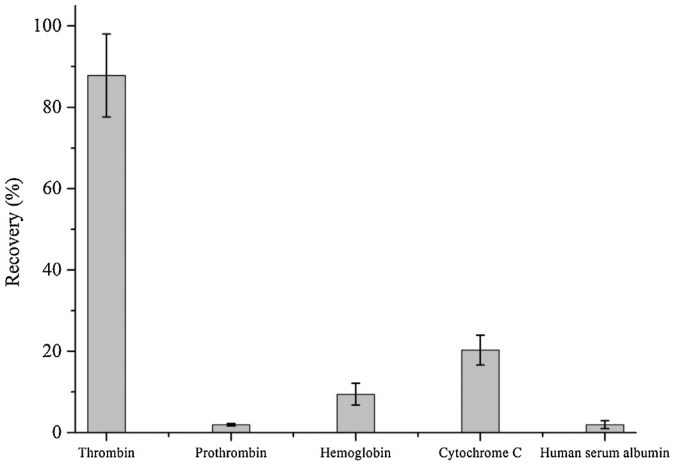



**Fig. 2.** Specificity of Apt-PANCMA probes for thrombin from the most potential interfering proteins spiked in PBS buffer (pH 7.4). The concentrations of α-thrombin, prothrombin, hemoglobin and human serum albumin were 5 nM. The concentration of cytochrome C was 10 nM. Total volume of the sample solution was 2 mL. Percent recovery was calculated from the calibration curves obtained by injecting different concentrations of standard proteins to the LC–MS/MS, as shown in the Table S-2.

developed LC–MS/MS method was 0.24 nM, which was evaluated on the basis of a signal-to-noise ratio of 3.

The reproducibility of the retention time and peak area were estimated by six repetitive determinations of the digestion solution from 5.0 nM of thrombin. The variation coefficients of retention time and the XIC peak area of the selected signal peptide were not larger than 1.0% and 5.7%, respectively. Results indicated that the repeatability of the LC–MS/MS method for determination of thrombin was satisfactory.

### *3.4. Evaluation of thrombin binding capacity, reproducibility, and stability of Apt-SPME probes*

In order to compare the specific extraction properties of the Apt-SPME probe, a binding assay was performed with thrombin spiked in either standard solution (PBS) or in diluted plasma, as shown in Fig. 4. Extraction profile in PBS indicates that thrombin can be extracted at a nearly quantitative yield at lower concentrations (below 10 nM); however, the extraction efficiency gets lower as concentration rises past 10 nM, and finally the probe reaches saturation at around 22 nM concentration. The maximum binding capacity of the prepared Apt-SPME probe for thrombin was estimated at approximately 20.7 pmol. The same experiment repeated in 20-fold diluted human plasma is shown in the filled symbols (Fig. 4). The experimental data indicates that specific capture and detection of thrombin from blood plasma is possible by Apt-SPME probe. For all concentration ranges, however, the extracted amount of thrombin from plasma is significantly lower than that obtained from PBS sample. In addition, the extraction isotherm in plasma shows a different saturation behavior; the maximum thrombin extraction capacity was almost half compared to the amount in PBS. First, this reduction of recovery was believed to be related to the inactivation of the specific aptamer probes by some other high abundant proteins. However, when this assumption was investigated by spiking increased levels of human serum albumin (HSA) up to the level of human blood (Fig. S-4), results showed that the recovery of thrombin was more than 60%, even if the concentration levels are the same as found in human blood. This indicates that the probe surface was quite functional and specific for thrombin even at high protein concentrations. It is worth mentioning that other research groups have also observed similar reductions of thrombin recovery from complex sample matrices. For instance, Yu's group [43] and Le's group [44] reported up to 80% thrombin recovery decreases in the 10-fold diluted serum compared to the buffer solution by using aptamer as aspecific receptor of thrombin. Reduction of signal intensity in a 10-fold diluted serum was also observed by Tok and co-workers [45], where they have used immobilized anti-thrombin antibodies as the specific probe. All of these studies indicate that the diminished thrombin recovery might be due to a decrease in functional thrombin concentration after spiking in the plasma/serum sample. This thrombin inactivity is probably due to the presence of thrombin inhibitors in serum or plasma. It has been reported that the most common enzymatic inactivation of thrombin occurs by forming an inactive complex with anti-thrombin III present in serum or plasma [46]. The results indicate that in order to extract intact thrombin with high recovery from plasma or serum by affinity ligands, it is necessary to indirectly protect inactivation of thrombin by spiking some artificial inhibitor so that the endogenous inactivation site is blocked while leaving the aptamer affinity site free for binding.

This approach of aptamer-based SPME can be miniaturized into high throughput 96-well format for quantitative proteomics. Furthermore, selective extraction of targeted low-abundance

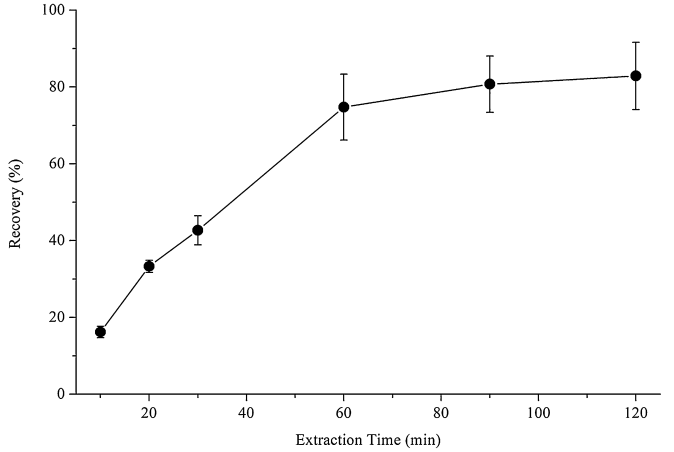



**Fig. 3.** Effect of extraction time on the recovery of thrombin from PBS buffer (pH 7.4). The concentration of thrombin was 5 nM and the volume of solution was 2 mL. Percent recovery was calculated from the calibration curves obtained by injecting different concentrations of standard proteins to the LC–MS/MS, as shown in Table S-2.

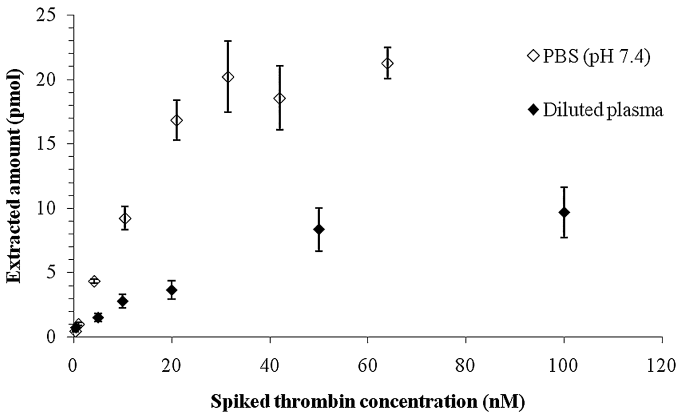



**Fig. 4.** Extraction efficiency of Apt-SPME probe for thrombin in standard solutions (PBS, pH 7.4) and in spiked diluted plasma samples. Thrombin was spiked in 2 mL of PBS or 20 fold diluted plasma and one Apt-SPME probe was incubated for 1 h at room temperature.

therapeutic or disease related proteins *in vivo* from human blood will be possible for detection with mass spectrometry.

Considering the effect of the plasma matrix on extraction performance, a calibration curve based on thrombin recovery was built to determine thrombin in human plasma. Fig. S-5(A) shows that the peak area of the signal peptide (ELLESYIDGR) at $m/z$ 839.38 from thrombin increased as the concentration of spiked thrombin increased from 0.5 to 150 nM, while that of the signal peptide did not change when the concentration of spiked thrombin was higher than 100 nM, resulting from the limited binding capacity of the Apt-PANCMA probe. As can be seen in Fig. S-5(B), the calibration curve was linear over the concentration range of 0.5–50 nM with a good correlation coefficient ($R^2 = 0.9923$), which indicated that the obtained calibration curve can be used to determine the concentration of thrombin in diluted human plasma, although the recoveries of thrombin from 20-fold diluted human plasma were not high. According to the above obtained results, the detection limit of the proposed Apt-PANCMA probe coupled with LC–MS/MS was found to be 0.30 nM, which was lower than 3.4 nM achieved by using aptamer functionalized organic–inorganic hybrid silica monolith – UV [33] and 16 nM obtained by paper electrochemical device [47], and some other LOD results [17]. In addition, the obtained LOD (0.30 nM) by using Apt-SPME–LC–MS/MS method for determination of real human plasma was similar to the LOD (0.24 nM) by using LC–MS/MS method for determination of standard thrombin solution, which suggests that the interference from other proteins was not obviousfor determination of thrombin after purification with the Apt-SPME, which suggested that the proposed Apt-SPME–LC–MS/MS method could be successfully applied to determine thrombin in clinical human plasma samples.

The probe-to-probe reproducibility was determined by evaluating the thrombin recovery on six different Apt-SPME probes. The obtained results showed that thrombin recoveries at a concentration level of 5.0 nM spiked in PBS buffer ranged from 87.8% to 103.2% for all six probes with the corresponding RSDs lower than 14.6%, which indicated that the Apt-SPME probe could be produced and operated reproducibly.

To investigate the stability of the Apt-SPME probe, the same probe was used for two days every week for one month. The RSD of the thrombin recovery at the same loading amounts was 11.7%, demonstrating that the Apt-SPME probe maintained good stability resulting from good mechanical stability of PANCMA microfibers on the probe and good intrinsic stability of aptamers conjugated on the surface of PANCMA. In addition, the thrombin recovery maintained about 80% after even 40 times reuse of the probes for the extraction of thrombin from diluted human plasma samples, which indicated that the Apt-SPME probe was stable and could be repeatedly used for more than 40 purification cycles in the extraction of analytes from real samples.

### 3.5. Application of the Apt-PANCMA-probe-LC–MS/MS to complex sample

The proposed Apt-SPME-probe coupled to LC–MS/MS was applied for the determination of thrombin in clinical human plasma samples. Considering the matrix effect of diluted human plasma on the determination of thrombin, the concentration of thrombin in human plasma samples was determined by the linear regression equation based on the thrombin recovery. Table 1 shows that thrombin concentrations were found to be different in different clinical human plasma samples. According to the results of Table 1, thrombin concentrations were about 248 and 285 nM in undiluted plasma samples before and during bypass surgery, respectively, which was beneficial to help control bleeding during bypass surgery [32]. These results suggested that the proposed Apt-SPME-probe-LC–MS/MS method was valid in the determination of thrombin in human plasma samples.

**Table 1**
Results of thrombin concentration in selected human plasma samples ($n = 3$).[a]

| Sample | Detected concentration in the 20-fold diluted plasma sample (nM) |
|---|---|
| Human plasma (before bypass surgery) | $12.38 \pm 0.87$ |
| Human plasma (during bypass surgery) | $14.23 \pm 0.62$ |
| Human plasma (after bypass surgery) | $4.09 \pm 0.41$ |
| Human plasma from LBL Inc. | —[b] |

[a] *Note*: The detected concentration was calculated by the calibration curve based on the recovery of thrombin (see Fig. S-7).
[b] "—" means "not detected".

## 4. Conclusions

A novel selective SPME probe based on the covalent immobilization of aptamers on electrospun PANCMA micro-fiber was developed to enrich thrombin from human plasma samples. The porous surface morphology of the polymeric substrate is important for large molecule extraction. Due to high surface area and high selective recognition, the prepared Apt-SPME probe has been shown to be a suitable selective SPME coating for extracting trace thrombin from samples. Under optimal conditions, the Apt-SPME probes were used to directly extract thrombin from diluted human plasma without any further sample preparation, and were stable enough for more than 40 replicate extraction cycles in the analysis of real samples. It should be noted that the porosity of the coating can also provide a negative impact on the performance by retaining unwanted species from the sample matrix. The employed LC–MS/MS analysis aided in the improvement of the sensitivity and accuracy of the whole assay, enabling the identification and quantification of trace thrombin from complex clinical samples. The proposed Apt-SPME probe coupled with LC–MS/MS method was successfully applied for the determination of thrombin in human plasma samples, which demonstrated that the proposed method is a promising technique for selective determination of trace proteins in clinical biological matrices. Upon further optimization of the probe in terms of matrix effects and biocompatibility, this approach can be used for *in vivo* protein extraction from human tissue and blood. Moreover, this selective SPME can be combined directly with mass spectrometry to provide a simple, high throughput diagnostic tool in biomedicine that eliminates most matrix interferences from body fluids without the need for time-consuming chromatography steps. Our current research effort is to improve the surface morphology of the substrate by using different polymer chemistries to obtain a robust coating for *in vivo* studies.

## Conflict of interest

The authors declare no competing financial interests.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.08.018.

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