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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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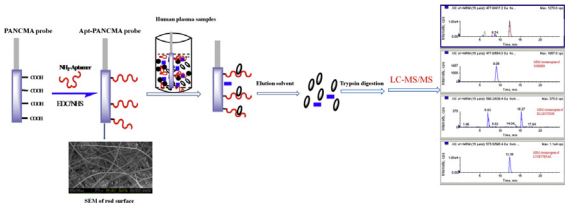
HIGHLIGHTS

- 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.