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Aptamer-based organic-silica hybrid affinity monolith prepared via "thiol-ene" click reaction for extraction of thrombin

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ABSTRACT

A novel strategy for preparing aptamer-based organic-silica hybrid monolithic column was developed via "thiol-ene" click chemistry. Due to the large specific surface area of the hybrid matrix and the simplicity, rapidness and high efficiency of "thiol-ene" click reaction, the average coverage density of aptamer on the organic-silica hybrid monolith reached 420 pmol μL^{-1}. Human α-thrombin can be captured on the prepared affinity monolithic column with high specificity and eluted by $NaClO_4$ solution. *N-p*-tosyl-Gly-Pro-Arg *p*-nitroanilide acetate was used as the sensitive chromogenic substrate of thrombin. The thrombin enriched by this affinity column was detected with a detection of limit of 0.01 μM by spectrophotometry. Furthermore, the extraction recovery of thrombin at 0.15 μM in human serum was 91.8% with a relative standard deviation of 4.0%. These results indicated that "thiol-ene" click chemistry provided a promising technique to immobilize aptamer on organic–inorganic hybrid monolith and the easily-assembled affinity monolithic material could be used to realize highly selective recognition of trace proteins.

1. Introduction

Aptamers are the artificial single-stranded DNA or RNA sequences generally selected using SELEX (Systematic evolution of ligands by exponential enrichment) [1,2] and can specially bind to molecular targets with high selectivity and affinity. Because of their advantages over antibodies such as easier modification and immobilization, better stability and higher reproducibility, aptamers have been used as affinity ligands [3,4] on stationary phases in affinity capillary chromatography to achieve the separation and quantification of targets.

At present, three categories of capillary columns, including packed columns [5,6], open tubular capillaries [7] and monolithic columns [8–10] have been employed for aptamer immobilization, among which organic–inorganic hybrid monolithic columns prepared by sol–gel method are favored due to the merits such as large specific surface area, good biocompatibility and high mechanical stability. Based on the nature of aptamers and monolithic columns, many approaches for immobilizing aptamers have been developed mainly including avidin–biotin interactions [11] and chemical covalent coupling [12]. As an example, Zhao et al. [10] decorated biotinylated DNA aptamer on streptavidin modified polymer monolithic capillary column. In view of the fact that various functional groups (such as $-NH_2$, –SH, –COOH, etc.) can be introduced at the end of aptamers easily, several chemical methods have been used to immobilize aptamers on monolithic columns. Wang et al. [8] covalently bound anti-lysozyme DNA aptamer on poly (glycidyl methacrylate-co-ethylene dimethacrylate) monolithic column and realized selective extraction and screening of a basic protein lysozyme with the aptamer-based high performance affinity chromatography. Zhang et al. [13] reported the successful immobilization of 5′-NH_2 modified aptamer on tetraethoxysilane-3-aminopropyltriethoxysilane (TEOS-3-APTMS) hybrid monolithic column for highly selective recognition of thrombin. However, the procedures of assembling aptamers above are somewhat tedious and time-consuming, and the average coverage densities of aptamers are limited on the hybrid monoliths.

Since first introduced by Sharpless et al. [14], click chemistry has attracted a lot of interest and importance in recent years. Owing to the toxicity of copper (I)-catalyzed alkyne–azide cycloaddition (CuAAC), "thiol-ene" click reaction with characteristics of simplicity, mild process and high yield has been widely applied in the functionalization of surfaces and stationary phases. Feng et al. [15] implemented "thiol-ene" click chemistry to prepare a Thiol-Click-COOH column. This newly developed column had a higher surface charge density and stronger hydrophilicity than pure silica column and achieved a good separation of 13 nucleosides, base and four

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water-soluble vitamins. Also, Yao et al. [16] reported a new strategy for the preparation of monolithic trypsin microreactor by "thiol-ene" click chemistry. Recently, Zhang et al. [17] developed a new approach to prepare organic–inorganic hybrid boric acid affinity monolith via "thiol-ene" click reaction and further applied to the selective capture of glycoproteins ovalbumin and horseradish peroxidase. Although covalent attachment based on click chemistry was performed for enzymes [18,19] and proteins [20], very few studies have been conducted to immobilize aptamers on monolithic columns by this facile reaction up to now.

Thrombin is a common protein that catalyzes many coagulation-related reactions responsible for blood clotting and can be used as a therapeutic and a biomarker for diagnosis of some diseases, such as pulmonary metastasis, diseases associated with coagulation abnormalities, and synovial inflammation [21,22]. Therefore, a sensitive and specific analytical method is necessary for thrombin. Numerous assays and biosensors have been exploited for thrombin using aptamers, by measuring electrochemical and optical signals, etc [23–27]. However, most of the aptamer-based strategies require redundant labeling or conjugation steps. It is thus highly desirable to develop simple and label-free techniques for aptamer-based analysis of thrombin. Taking advantage of the activity of thrombin toward small-molecule substrates, aptamer-capture based methods have been proposed for the determination of thrombin. Mir et al. [28] indicated that it was possible to assay captured thrombin by detecting the thrombin reaction products either optically or electrochemically, though the reported sensitivity was a bit low. Centi et al. [29] developed the analysis of thrombin using aptamers and magnetic beads by electrochemically measuring the enzymatic product of thrombin, and the limit of detection (LOD) for thrombin was about 175 nM. Zhao et al. [30,31] proposed aptamer-capture based assays for thrombin by using magnetic beads or microplates and fluorescently measuring the products of the catalyzed reactions, and the detection of 2 to tens fM thrombin could be achieved. In our previous report [27], an aptamer-based protocol was developed for 0.4 fmol thrombin on magnetic beads with fluorescence detection using a microarray scanner with only 1 microliter solution consumption.

In this present work, a novel approach of the covalent immobilization of DNA aptamers on organic-silica hybrid monolith was proposed via facile "thiol-ene" click chemistry for the preparation of aptamer-based affinity monolithic column, through which the average coverage density and binding capacity of aptamer were both increased. Meanwhile, a simple spectrophotometric method using *N*-*p*-tosyl-Gly-Pro-Arg *p*-nitroanilide acetate (T1637) as chromogenic substrate [32] was employed for human thrombin enriched by the aptamer affinity column. By taking advantage of analyte enrichment and enzyme amplification, this assay showed excellent specificity and comparable sensitivity by binding of aptamer to the affinity column and special enzyme reaction. Also, an efficient extraction of thrombin in spiked human serum was realized with satisfactory recovery, indicating the great potential of such affinity columns for trace proteins detection in complex matrix.

2. Experimental

2.1. Chemicals and materials

Tetramethoxysilane (TMOS, 98%) and 2,2′-azobis (2-methylpropionamidine) dihydrochloride (V50) were both purchased from J & K Technology Co. Ltd. (Shanghai, China). γ-Methyl methacrylate trimethoxysilane (γ-MAPS, 98%) was obtained from Sigma-Aldrich Co. (Shanghai, China). Polyethylene glycol (PEG, MW=6000 (6 K), 10,000 (10 K), 20,000 (20 K)) was purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Sodium perchlorate ($NaClO_4$) and acetic acid (HAc) were obtained from National Chemical Reagent Co. (Nanjing, China). Tris (hydroxymethyl) aminomethane (Tris) was purchased from Biosharp Co. (Seoul, Korea). Dithiothreitol (DTT) was obtained from Bio-Rad Co. (USA). All other chemicals were analytical grade. Deionized water (DIW, 18.25 MΩ cm) prepared from a Milli-Q water system (Millipore, Bedford, MA, USA) was used throughout the experiment.

Human α-thrombin and its chromogenic substrate *N*-*p*-tosyl-Gly-Pro-Arg *p*-nitroanilide acetate (T1637) were both obtained from Sigma Aldrich (Shanghai, China). Bovine serum albumin (BSA), human serum albumin (HSA), human immunoglobulin (IgG) and lysozyme (Lyz) were purchased from Sengon Biotech. Co. (Shanghai, China). The aptamer against human α-thrombin (5′-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′, Apt29) and DNA oligo control poly A29 (5′-AAA AAA AAA AAA AAA AAA AAA AAA AAA AA-3′, A29), both with 5′-end modified by –SH through a C6-carbon spacer arm, were synthesized and purified by Sangon Biotech. Co. (Shanghai, China).

The oligonucleotides stock solutions (100 μM) were respectively prepared in 10 mM Tris–HCl buffer solution (1 mM KCl, 140 mM NaCl, pH 7.4) and stored at −20 °C. Human α-thrombin was prepared into 3.0 μM aqueous stock solution, and subsequently divided into 10 μL in every 0.5 mL centrifuge tube and stored at −20 °C. BSA was prepared into 0.1 mg mL^{-1} aqueous working solutions. Other proteins were respectively prepared into 1.0 mg mL^{-1} aqueous working solutions.

2.2. Preparation of aptamer-based organic-silica hybrid monolithic column

The fused-silica capillary (530 μm i.d., Reafine Chromatography Ltd., Hebei, China) was used to prepare the monolithic column. Prior to preparation, the capillary was activated by 1.0 M sodium hydroxide

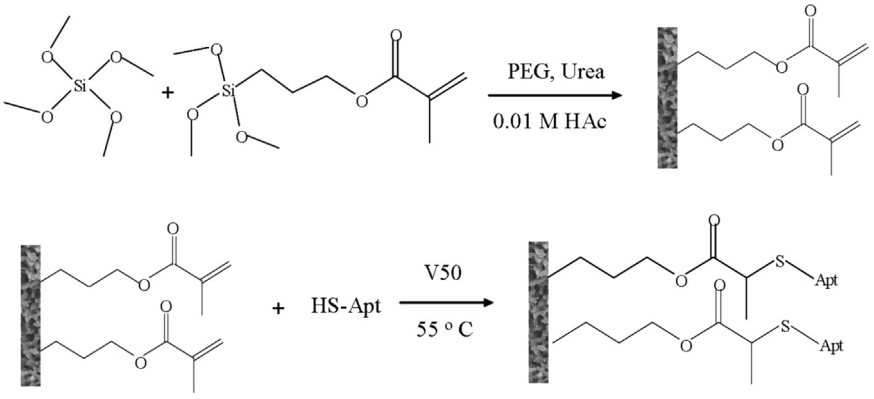

Fig. 1. Scheme for preparation of aptamer-based hybrid monolithic column via "thiol-ene" click chemistry.