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A rapid sandwich immunoassay for human fetuin A using agarose-3-aminopropyltriethoxysilane modified microtiter plate

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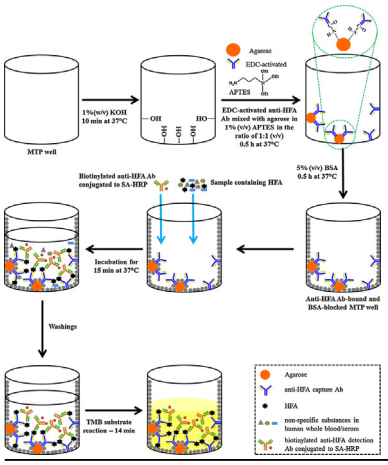
HIGHLIGHTS

- Agarose-based signal enhanced immunoassay for human fetuin A (HFA).
- Wide linear range of 1–243 ng mL^{-1} with high sensitivity and specificity.
- Limit of detection of 0.02 ng mL^{-1} and limit of quantification of 0.3 ng mL^{-1}.
- Detection of clinically-relevant HFA levels in human blood and serum in <30 min.
- High analytical precision and ~14-fold faster than conventional ELISA.

GRAPHICAL ABSTRACT



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ABSTRACT

A rapid sandwich immunoassay (IA) with enhanced signal response for human fetuin A (HFA) was developed by modifying the surface of a KOH-treated polystyrene microtiter plate (MTP) with agarose and 3-aminopropyltriethoxysilane (APTES). The agarose-APTES complex binds covalently to the hydroxyl moiety of the MTP plate to serve as a binding platform for bioconjugation of EDC-activated anti-HFA antibody (Ab) via carbodiimide coupling. The one-step kinetics-based sandwich enzyme-linked immunosorbent assay (ELISA) enabled the detection of HFA in 30 min with a limit of detection (LOD) and a linear range of 0.02 ng mL^{-1} and 1–243 ng mL^{-1}, respectively. It detected HFA spiked in diluted human whole blood and serum, and HFA in ethylenediaminetetraacetic acid (EDTA)-plasma of patients with high precision similar to that of conventional ELISA. The anti-HFA Ab-bound agarose-functionalized MTPs retained their functional activity after 6 weeks of storage in 0.1 M PBS, pH 7.4 at 4 °C.

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1. Introduction

Human fetuin A (HFA) plays an important role in various physiological and pathophysiological processes [1]. It is a specific biomarker of hepatocellular carcinoma [2,3] and atherosclerosis [4–6], and is associated with a plethora of diseases/disorders such as diabetes [7–16], cardiovascular diseases [17–23], metabolic syndrome [24,25], obesity [26], multiple sclerosis [27], pancreatic cancer [28], breast cancer [29], malaria [30], alcoholic liver cirrhosis [2,31], kidney malfunction [32] and arthritis [33]. It is produced by the liver and plays an anti-inflammatory role by counteracting the production of proinflammatory cytokines [34,35]. As a negative acute-phase reactant, HFA concentrations in serum decrease during the inflammatory acute phase reaction [36]. HFA might contribute to the formation of mineral nanoparticles (NPs) in patients having calcification of arteries [37], with the HFA content being dependent on the presence of calcium and phosphate ions. This could be the rationale behind a drastic decrease in HFA concentrations in the presence of calcification precursors found in patients with cardiovascular diseases (CVD) [38,39], atherosclerosis [19] and patients on dialysis [32]. Decreased HFA levels in serum are also observed in acute alcoholic hepatitis, chronic autoimmune hepatitis, fatty liver, alcoholic and primary biliary cirrhosis, and hepatocellular carcinoma [40]. The selective HFA contribution in the NPs, with a high potential to promote inflammation as opposed to its anti-inflammatory function, is of major relevance. The determination of HFA with high sensitivity is useful in the assessment of neuroinflammation [41] and multiple sclerosis [27].

ELISA is a highly-sensitive IA format for the detection of HFA [42–44] with a total analysis time of several hours. Accordingly, different prospective IA formats have been attempted [30,45,46], including the microfluidics-based rapid IA using surface plasmon resonance (SPR) with the IA duration of ~10 min [47,48]. However, the SPR-based IA is not as high-throughput and sensitive as ELISA-based formats, and requires a costly and disposable SPR chip (over 100 USD) together with expensive instrumentation. In addition, in an extended meta-analysis on the clinical value of HFA quantification in different diseases, study results are in part contradictory [20].

This study describes a rapid and simple sandwich IA format with enhanced signal response for the detection of HFA in diluted human whole blood and serum within 30 min (Fig. 1). The signal enhancement is achieved using agarose microparticles modified with APTES, which provides increased surface area for a significantly high capture HFA Ab loading. Albeit agarose has been used extensively as an immobilization matrix for enzymes/proteins, its application in immunoassay has not been attempted. The assay format offers a significantly reduced IA duration with minimal process steps compared to the conventional sandwich ELISA (cELISA). The analytical performance of the developed IA (DIA) will be evaluated for total analysis time, detection limit, linear range and selectivity, and validated with cELISA.

2. Materials and methods

2.1. Materials

Phosphate buffered saline (PBS, 0.1 M, pH 7.4), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), bovine serum albumin (BSA), 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 4.7), and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit were purchased from Thermo Scientific. Agarose (~85 μm in diameter), 3-aminopropyltriethoxysilane (APTES, purity 98%, w/v), H_2SO_4 (97.5%, v/v), and Nunc microwell 96-well polystyrene plates (flat bottom, non-treated and sterile) were procured from Sigma–Aldrich. The HFA kit, consisting of mouse anti-HFA capture Ab, recombinant HFA, biotinylated goat anti-HFA detection Ab and streptavidin-conjugated horseradish peroxidase (SA-HRP), was obtained from R&D Systems, USA. Human whole blood (HQ-Chex Level 2) and human serum were purchased from Streck, USA and HyTest Ltd., Finland, respectively. The anonymized EDTA plasma samples with various HFA levels were provided by the University Hospital Ulm, Germany. Human serum albumin (HSA), C-reactive protein (CRP), human lipocalin-2 (LCN2), interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α are the products of R&D Systems, USA. The assay components were reconstituted in 0.1 M PBS with 0.1% (v/v) BSA, while all buffers and solutions were prepared with ultrapure water (UPW, 18 MΩ, Direct Q, Millipore, USA). The dilutions of HFA assay components and BSA were made in 0.1 M PBS, whereas KOH and 3-APTES were diluted in UPW. Agarose (1 mg) was mixed with 1 mL of 1% APTES followed by ultrasonication for 1 h before use. The HFA concentrations were prepared in BSA-preblocked sample vials to minimize the analyte loss due to its non-specific binding [49]. The HFA-spiked samples were obtained by spiking various HFA concentrations in a fixed dilution (1:100) of human whole blood and plasma. The clinical samples containing HFA from 1 to 200 μg mL^{-1} and >200 μg mL^{-1} were diluted 1:1000 and 1:3000, respectively, to fit the linear range of DIA (1–243 ng mL^{-1}). Both dilutions of 1:1000 and 1:3000 were employed for each of the unknown clinical samples, as per the standard sample preparation guidelines for the clinical ELISA. The assay was performed at 37 °C (Thermostat, Labnet International, USA), while the absorbance was measured by the Tecan Infinite M200 Pro microplate reader from Tecan (Austria). DIW and PBS washings were performed five times with 300 μL of each solution, while the blocking was done with 300 μL of 5% BSA. Similarly, 100 μL was taken for the dispensing of 1% KOH, EDC-activated capture anti-HFA Ab mixed with agarose in 1% (v/v) APTES, HFA, biotinylated anti-HFA Ab conjugated to SA-HRP, and TMB. EDC was reconstituted in 0.1 M MES. The biotinylated anti-HFA detection Ab (0.2 μg mL^{-1}) was admixed and incubated with SA-HRP (diluted 1:200), 1:1 (v/v), resulting in the conjugation of biotinylated anti-HFA detection Ab to SA-HRP.

2.2. Preparation of anti-HFA Ab-bound agarose-functionalized MTP

The treatment of the MTP wells with 1% (w/v) KOH at RT for 10 min followed by washing with UPW generates the desired hydroxyl groups for subsequent binding of APTES (Fig. 1). Unless otherwise indicated, the following steps were performed at 37 °C. EDC-activated anti-HFA Ab was prepared by incubating 990 μL of anti-HFA Ab (8 μg mL^{-1}) with 10 μL of EDC (4 mg mL^{-1}) for 15 min. The EDC-activated anti-HFA Ab was then mixed with agarose (1 mg mL^{-1}) in 1% (v/v) APTES in the ratio of 1:1 (v/v). Thereafter, 100 μL of this anti-HFA Ab solution (4 μg mL^{-1}, 0.5 mg mL^{-1} agarose and 0.5% APTES) was dispensed into each well, incubated for 30 min and washed with PBS. The anti-HFA Ab-bound agarose-functionalized wells were blocked with 5% (v/v) BSA for 30 min and washed with PBS. The stability of anti-HFA Ab-bound and BSA-preblocked MTPs, stored in 0.1 M PBS at 4 °C, was assessed for 6 weeks.

2.3. Developed sandwich IA procedure

The anti-HFA Ab-bound and BSA-preblocked MTP was provided with biotinylated anti-HFA Ab preconjugated to SA-HRP, and HFA (0.1–243 ng mL^{-1}) in buffer, diluted human serum or diluted human whole blood. It was then incubated for 15 min and subsequently washed with PBS. This was followed by the addition of TMB (as per manufacturer's guidelines) and stopping of the enzyme–substrate reaction after 14 min by adding 50 μL of 2N