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Development of a High Sensitivity Rapid Sandwich ELISA Procedure and Its Comparison with the Conventional Approach

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A highly sensitive and rapid sandwich enzyme-linked immunosorbent assay (ELISA) procedure was developed for the detection of human fetuin A/AHSG (α2-HS-glycoprotein), a specific biomarker for hepatocellular carcinoma and atherosclerosis. Anti-human fetuin A antibody was immobilized on aminopropyltriethoxysilane-mediated amine-functionalized microtiter plates using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide-based heterobifunctional cross-linking. The analytical sensitivity of the developed assay was 39 pg/mL, compared to 625 pg/mL for the conventional assay. The generic nature of the developed procedure was demonstrated by performing human fetuin A assays on different polymeric matrixes, i.e., polystyrene, poly(methyl methacrylate), and polycyclo-olefin (Zeonex), in a modified microtiter plate format. Thus, the newly developed procedure has considerable advantages over the existing method.

Conventional enzyme-linked immunosorbent assay (ELISA) procedures have been followed for decades for the detection of analytes of importance in industrial, healthcare, and academic research. However, improvement on existing ELISA technologies are continuously attempted by many groups.[1,2] We report the development of a high-sensitivity ELISA-based assay for human fetuin A (HFA), with lower detection limits and a higher sensitivity than commercially available assays. The biological importance of HFA, a member of the cystatin superfamily, which is commonly present in the cortical plate of the immature cerebral cortex and hemopoietic matrix of bone marrow, is discussed elsewhere.[3−10] There are many commercially available ELISA kits for fetuin A, which are listed in Supplementary Table 1, Supporting Information. The assay was demonstrated on different commercially relevant solid supports. The developed ELISA is better than the conventional procedure in terms of greatly reduced overall assay duration, higher sensitivity, and greater reproducibility.

HFA was taken as the model assay system to demonstrate the utility of our developed ELISA procedure since all the assay components were commercially available in kit form. This enabled us to do robust and highly precise comparison of the developed ELISA with the commercially existing conventional ELISA procedures, as the same assay components were used under the same conditions. The developed procedure can be employed on any commercially relevant substrate. Therefore, this approach of immobilizing antibody on chemically modified solid supports (Figure 1) has potential applications in many other assays and formats.

EXPERIMENTAL SECTION

Plate Preparation, Amine-Functionalization with Silane, and Cross-Linking Carboxyl Groups of Anti-HFA Antibody to the Amino Groups of the Surface of Microtiter Plate Wells. Each well of the 96-well plate was treated with 100 μL of absolute ethanol for 5 min at 37 °C and washed five times with 300 μL of deionized water (DIW). Subsequently, each well was treated with

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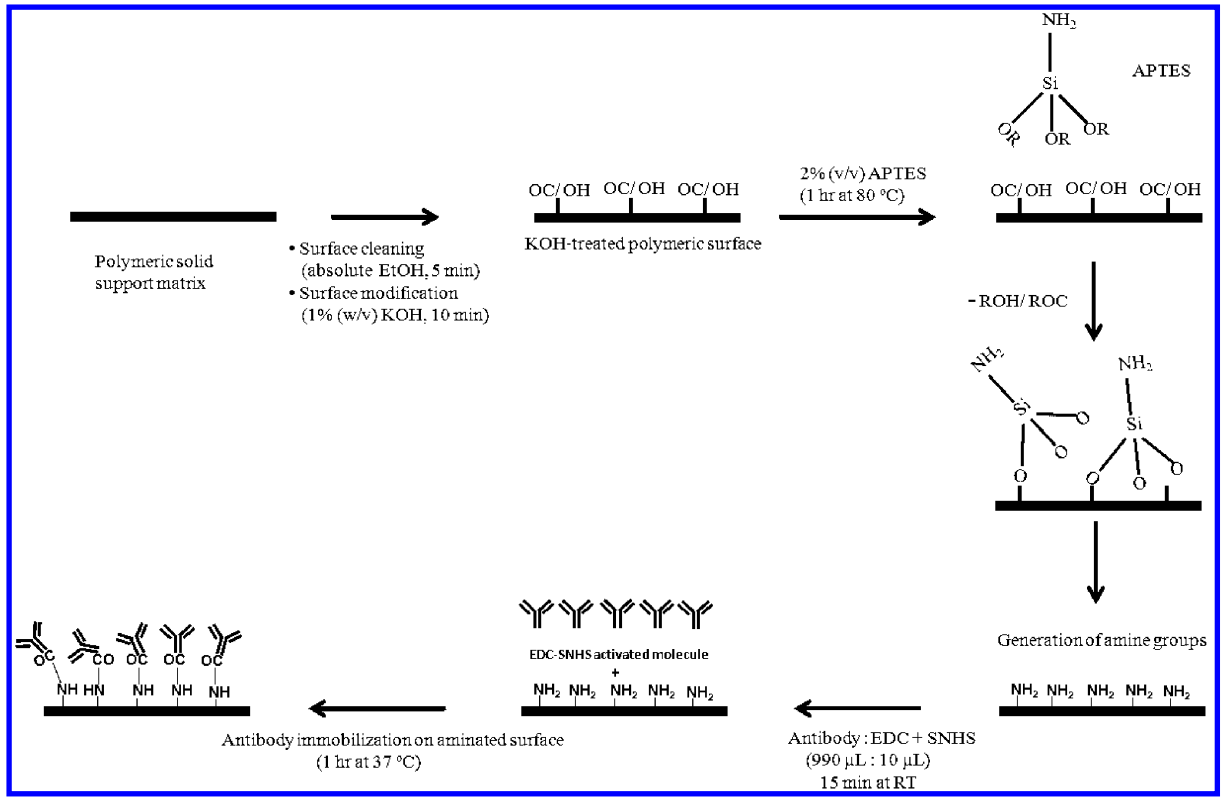

Figure 1. Schematic of the developed sandwich ELISA procedure for the HFA/AHSG assay. Plate functionalization was performed by treatment with 1% (w/v) KOH and then silanization with 2% (v/v) APTES. KOH oxidizes the organic moiety of the polymer generating a carbonyl (−CO) or a hydroxyl (−OH) group according to the nature of the polymer. The alkoxy groups of APTES subsequently react with the carbonyl or hydroxyl groups of the surface in a hydrolysis-dependent reaction.[11,12] Anti-HFA/AHSG antibody was then captured using EDC and sulfo-NHS chemistry on the aminated 96-well plate, where the EDC-SNHS was used in a ratio of 1:100 with the antibody. The EDC-SNHS-activated antibody molecules were then immobilized on the aminated ELISA plate, where a bond was formed between amine groups on the plate and the activated carboxyl groups of the antibody.

100 µL of 1.0% (w/v) KOH at 37 °C for 10 min followed by five washings with 300 µL of DIW. The KOH-treated wells were then functionalized with amino groups by incubation with 100 µL of 2% (v/v) aminopropyltriethoxysilane (APTES) per well at 80 °C for 1 h inside a vacuum-desiccator, in order to achieve maximum silanization. The reaction mechanism following the KOH activation and APTES-based surface functionalization involves mild oxidation followed by a hydrolytic APTES polymerization on the oxidized surface through its alkoxy groups.[11,12] The desiccator was equilibrated to room temperature for 20 min. The amine-functionalized plate was subsequently washed five times with 300 µL of DIW in order to remove excess unbound 3-APTES from the surface. Afterward, the anti-HFA/AHSG (α2-HS-glycoprotein; 990 µL of 4 µg/mL) was incubated with 10 µL of a premixed solution of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (4 mg/mL) and *N*-hydroxysulfosuccinimide (SNHS) (11 mg/mL) for 15 min at 37 °C. The resulting EDC cross-linked anti-HFA/AHSG antibody solution was added to each of the functionalized wells (100 µL) and incubated for 1 h at 37 °C. The anti-HFA/AHSG-coated wells were then washed five times with 300 µL of PBS. The methodology pertaining to the conventional assay is described in detail in the Supporting Information methods section.

ELISA on Anti-HFA/AHSG Antibody-Immobilized Microtiter Plates. Plates with covalent and passively immobilized anti-HFA antibody were blocked with 1% (v/v) BSA diluted in 0.1 M phosphate buffered saline (PBS), pH 7.4, for 30 min at 37 °C and subsequently washed five times with 300 µL of PBS. Varying concentrations of HFA/AHSG (from 4.8 pg/mL to 20 ng/mL) were prepared in 0.1 M PBS, pH 7.4, and 100 µL of each of these concentrations were incubated in the antibody-coated plates for 1 h at 37 °C and, subsequently washed five times with PBS. One hundred microliters of biotinylated anti-HFA/AHSG detection antibody (200 ng/mL) was added and incubated for 1 h at 37 °C followed by five PBS washes. HRP-conjugated streptavidin (100 µL per well), at a dilution of 1:200, was added and then incubated for 20 min at 37 °C followed by five washes with PBS. The 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was subsequently added (as per the manufacturer's recommendations), and the reaction was stopped after 20 min by addition of 50 µL of 1N H_2SO_4. The absorbance was recorded at a primary wavelength of 450 nm with a reference wavelength of 540 nm. The dual wavelength system of absorbance measurement in 96-well plates is designed to eliminate or greatly reduce the optical imperfections at the well-to-well level.

All the experiments were carried out in triplicate. The control for this study was 0 ng/mL HFA in 0.1 M PBS, pH 7.4, and the absorbance of the control was subtracted from all the assay values. The respective analytical sensitivity (detection limit) of the assay was determined where analytical sensitivity was calculated using the formula [mean absorbance of blank +3σ (standard deviation of the blank)]. Additionally, data sets obtained from the modified

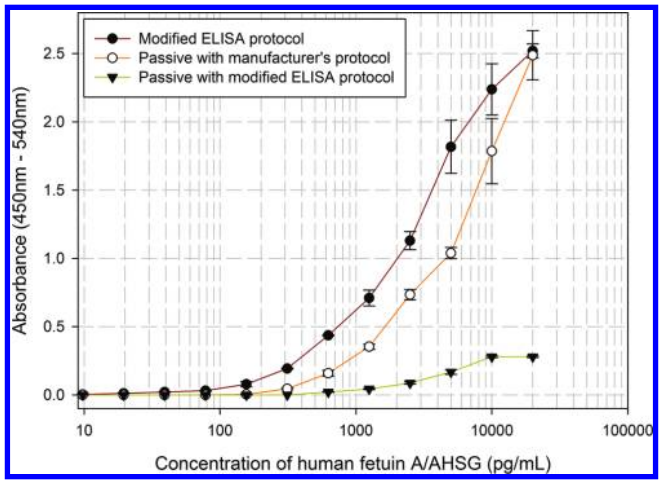

Figure 2. Comparative analysis of developed sandwich ELISA (black circles) with the conventional format. Anti-human fetuin A (anti-HFA) antibody was covalently bound to the amine-modified surface in the developed ELISA, whereas antibody adsorbed on the unmodified surface was used in the conventional assay format. The conventional assay was performed with the manufacturer-recommended protocol (white circles) and the protocol developed in this study (inverted triangle). The error bars represents standard deviations.

Table 1. Comparison of Assay Performance Parameters (Precision and Sensitivity) for Conventional and Developed ELISAs[a]

	intraday precision range (CV %), ($n = 5$)	interday precision range (CV %), ($n = 3$)	sensitivity (pg/mL)
developed ELISA	2.4−10.4	1.7−17.6	39
conventional ELISA	4.7−17.4	3.6−20.0	624

[a] Intraday precision was calculated from the five repeats ($n = 5$) of the same assay on a single day but at different times, while interday precision was calculated from assay repeats on three different days ($n = 3$). All the assays were carried out in triplicate. Analytical sensitivity was calculated using the formula [average absorbance of the blank + $3(SD_{blank})$].

and conventional ELISA were analyzed using standard curve analysis of Sigma Plot software, version 11.0.

RESULTS AND DISCUSSION

The range of detection of HFA/AHSG for the developed ELISA (Figure 2) was 9−20 pg/mL ($r^2 = 0.99$). An analytical sensitivity of 39 pg/mL was recorded for developed ELISA, where analytical sensitivity was calculated using the formula [average absorbance of the blank + $3(SD_{blank})$]. Assay variability parameters for this high-sensitivity assay for HFA are described in Table 1, where intraday assay variability was calculated from 5 repeats performed on a single day, and interday assay variability was calculated from three repeats performed on three different days in triplicate. The percentage recovery for lower concentrations (4.88−625 pg/mL) was 50−80% while for higher concentrations (1.2−20 ng/mL) it was 75−100%. The percentage recovery was calculated as the obtained value/expected value × 100.

The half-effective concentration (EC_{50}) obtained from the dose−response curve,[13] which is a measure of analyte−ligand interaction, was found to be on an average of 3.3 ng/mL. The EC_{50} for all the assay repeats was found to be in the range of 3−3.5 ng/mL. A lower EC_{50} (3.3 ng/mL), which was obtained for the developed ELISA, suggests a strong interaction between HFA and anti-HFA antibody. This interaction behavior is attributed to the significant increase in the total amount of immobilized antibody achieved using the covalent immobilization strategy, which increased the availability of anti-HFA antibody per HFA molecule, thus increasing the resultant HFA capture on the well surface.

Conversely, EC_{50} for the conventional ELISA format was 23 ng/mL, ($r^2 = 0.99$), which was significantly higher than the developed ELISA. This suggests a less sensitive conventional assay (Supplementary Table 2, Supporting Information). The most important factor that may be attributed to the enhanced sensitivity of the modified assay is the covalent immobilization of the anti-HFA/AHSG capture antibody since the covalently cross-linked antibodies should not leach out during the assay procedure in comparison to the use of passively adsorbed antibodies, where leaching may occur more easily.

Performance of the developed sandwich ELISA procedure was compared with the conventional protocols (Figure 2), both involving passively adsorbed antibody on normal unmodified microtiter plates (one performed using the modified protocol and the other as per the manufacturer's recommendations). All the assays were performed simultaneously on the same day under same set of conditions in order to minimize variability, where variability was reported as percentage coefficient of variation (% CV) (Table 1).

The technique employed here for chemically modifying the polymeric surface was developed and standardized by our group, and a standard EDC-based cross-linking strategy was deployed to immobilize anti-HFA/AHSG antibody. The developed sandwich ELISA procedure decreased the overall assay duration from 20 to 6 h, which is more than a 3-fold decrease. Thus, the reported procedure is a rapid ELISA format having the additional benefit of being generic in nature, i.e., it can be used with sandwich ELISAs on different polymeric matrixes for a range of different analytes. Hence, using the surface modification technique reported here, it is possible to develop rapid and high sensitivity assays on various categories of solid supports including those that are chemically inert.

To the best of our knowledge, this developed ELISA procedure is the most sensitive assay reported for the detection of HFA/AHSG (Supplementary Table 1, Supporting Information), which is based on the comparison between different commercially available ELISA kits for HFA. The sensitivity of the assay developed by our group may be attributed to the use of monoclonal antibody at the capture stage, which was covalently immobilized on the surface, and a polyclonal antibody to detect the antigen. The covalently cross-linked monoclonal antibodies that are used as capture antibodies in this study were found to capture significantly higher amounts of HFA, which has improved the detection limit and the overall assay sensitivity of the developed ELISA procedure in comparison to the conventional format. However, the commercially available ELISA kits, as described in Supplementary Table 1 (Supporting Information), have different capture and detection antibody combinations, which

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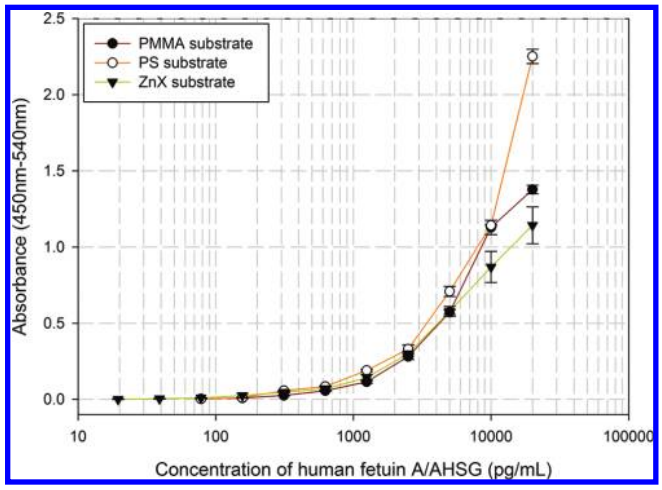

Figure 3. Performance of the developed ELISA on different solid support matrixes. Three categories of the solid support were chosen on the basis of their chemical properties, namely polystyrene (PS) which is hydrophobic (white circles); polymethyl methacrylate (PMMA), a hydrophilic polymer (black circles); and polycyclo-olefin polymer, trade name Zeonex, which is a neutral matrix (inverted black triangles). The error bars represent standard deviations.

may be a factor in their reduced sensitivities.[14] The sensitivity and specificity of any immunoassay is dependent on the antigen capture efficiency of an antibody which is subsequently governed by the nature of antibody[15,16] and accounts for the different assay sensitivities of the commercially available kits employing different type of antibodies that are monoclonal or polyclonal at the capture stage in ELISA.

Three different types of commercially relevant substrates, polystyrene (PS) (hydrophobic), polymethyl methacrylate (PMMA) (hydrophilic), and cyclo-olefin polymer (Zeonex) (inert), were used in the modified microtiter plate format. The HFA assay was performed on these matrixes in the modified microtiter plate format. The HFA assays performed on different solid substrates could not be directly compared because of their different thicknesses. Theoretically, the optical path length contributes toward the absorbance (*A*). Therefore, a thick solid support increases the path length and, hence, significantly changes the absorbance. However, the generic nature of the developed ELISA procedure was successfully demonstrated (Figure 3). The HFA assay performed on the Zeonex support, which is an inert cyclic poly olefin derivative, had an analytical sensitivity of 19.5 pg/mL, while for the assay performed on PS and PMMA, it was 78 pg/mL. The analytical sensitivities for the HFA assay and the dose−response, which determines the slope-dependent linearity range of the assay, of HFA-anti-HFA for all the three solid supports used in this study suggests that this modified protocol increases the overall assay sensitivity and is not confined to the nature of the solid support used for capturing antibody.

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CONCLUSIONS

A rapid sandwich ELISA procedure was developed for the highly sensitive detection of HFA/AHSG. It was based on the covalent immobilization of anti-HFA capture antibody on a 3-APTES-functionalized microtiter plate. The developed ELISA has comparatively better analytical sensitivity (39 pg/mL) and less variability in interday and intraday assay repeats than the conventional format, which has an analytical sensitivity of 624 pg/mL. The surface chemistry developed for this study is generic and can be employed to activate polymer matrixes irrespective of their chemical nature as demonstrated on PS (hydrophobic), PMMA (hydrophilic), and polycyclo-olefin (inert). Therefore, this strategy can also be used for rapid assay development on various commercially relevant substrates and the screening of substrates for particular biosensor/diagnostic applications.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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