SUBJECT AREAS

Immunodiagnostics; *In vitro* diagnostics.

One-step antibody immobilization-based rapid and highly-sensitive sandwich ELISA procedure for potential *in vitro* diagnostics

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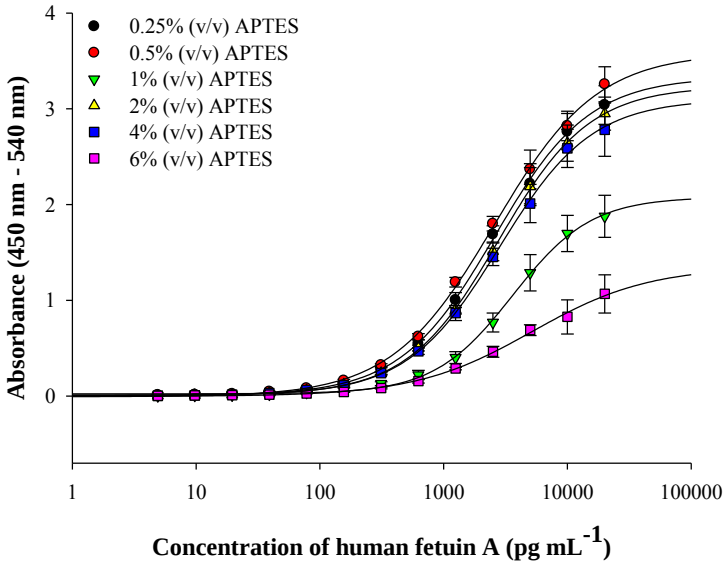
Materials

Phosphate buffered saline (PBS, 0.1 M, pH 7.4), 3,3',5,5'-tetramethylbenzidine (TMB), bovine-serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and bicinchoninic acid (BCA) protein assay kit were purchased from Thermo Scientific, while potassium hydroxide (KOH), 3-APTES, bovine immunoglobulin G (IgG) were obtained from Sigma-Aldrich. The human plasma and whole blood were procured from Biological Specialty Corp., USA and Streck, USA, respectively. The HFA/AHSG Duoset kit, containing all HFA assay components including mouse anti-HFA, recombinant HFA, biotinylated goat anti-HFA and streptavidin-conjugated horseradish peroxidase (SA-HRP), was procured from RnD Systems, USA. The thermostat was from Labnet International Inc., USA, while the Tecan Infinite M200 Pro microplate reader was from Tecan GmbH, Austria. Poly(methyl methacrylate) (PMMA), polystyrene (PS) and Zeonex^{TM} (Znx) slides were purchased from Microfluidic Chip Shop GmbH, Jena, Germany, while polycarbonate (PC) and cellulose acetate (CA) were from VTT, Finland, and Zeonor^{TM} (Znr) was from Zeon Chemicals, Germany. The pressure-sensitive adhesive (PSA) and bottomless 96-well ELISA plates were procured from Adhesive Research (Ireland) and Greiner Labortechnik (Germany), respectively.

(a)



(b)

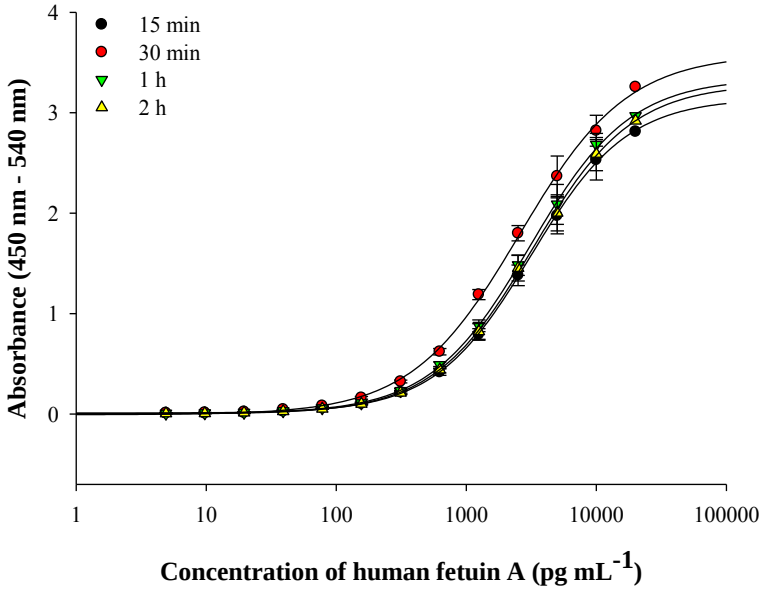


Figure S1. New immobilization format (NIF) for sandwich ELISA for HFA. (a) Optimization of the APTES concentration used for the dilution of anti-HFA. (b) Optimization of the duration of Ab immobilization. All experiments were done in triplicate, while the error bars represent standard deviations.

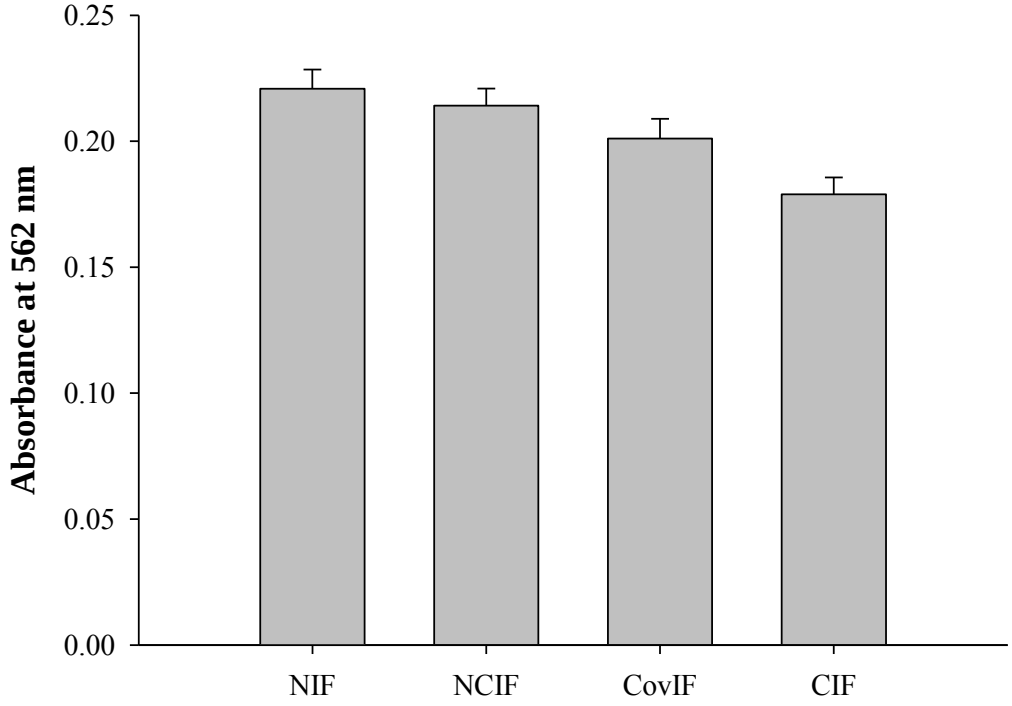


Figure S2. Determination of the anti-HFA immobilized on the MTPs, which were prepared using the new immobilization format (NIF), new covalent immobilization format (NCIF), our previously developed covalent immobilization format (CovIF)[1,2] and conventional immobilization format (CIF)-based sandwich ELISA procedures, by the bicinchoninic acid (BCA) protein assay. The NCIF-based sandwich ELISA involved the binding of EDC-activated anti-HFA diluted in APTES to the KOH-pretreated MTP. All experiments were done in triplicate with the error bars representing standard deviations.

Characterization of APTES functionalization and antibody binding to polystyrene

Experimental

Scanning electron microscope-energy dispersive X-ray (SEM-EDX) analysis was performed with a Hitachi S 2600N SEM (Hitachi Scientific Instruments, Tokyo, Japan) equipped with a microanalysis detector for EDX (Inca x-act, Oxford Analytical Instruments, Abington, UK). EDX

spectra were collected at 30º angle, 20 kV accelerating voltage and a 20 mm working distance. EDX results were analyzed using incorporated Inca, Point and Analyze software. Attenuated total reflectance FTIR (ATR-FTIR) spectra were collected from 4000 to 600 cm^{-1} for 64 scans and 4 cm^{-1} resolution using a zinc selenide (ZnSe) crystal on a Bruker Tensor 27 FTIR spectrophotometer.

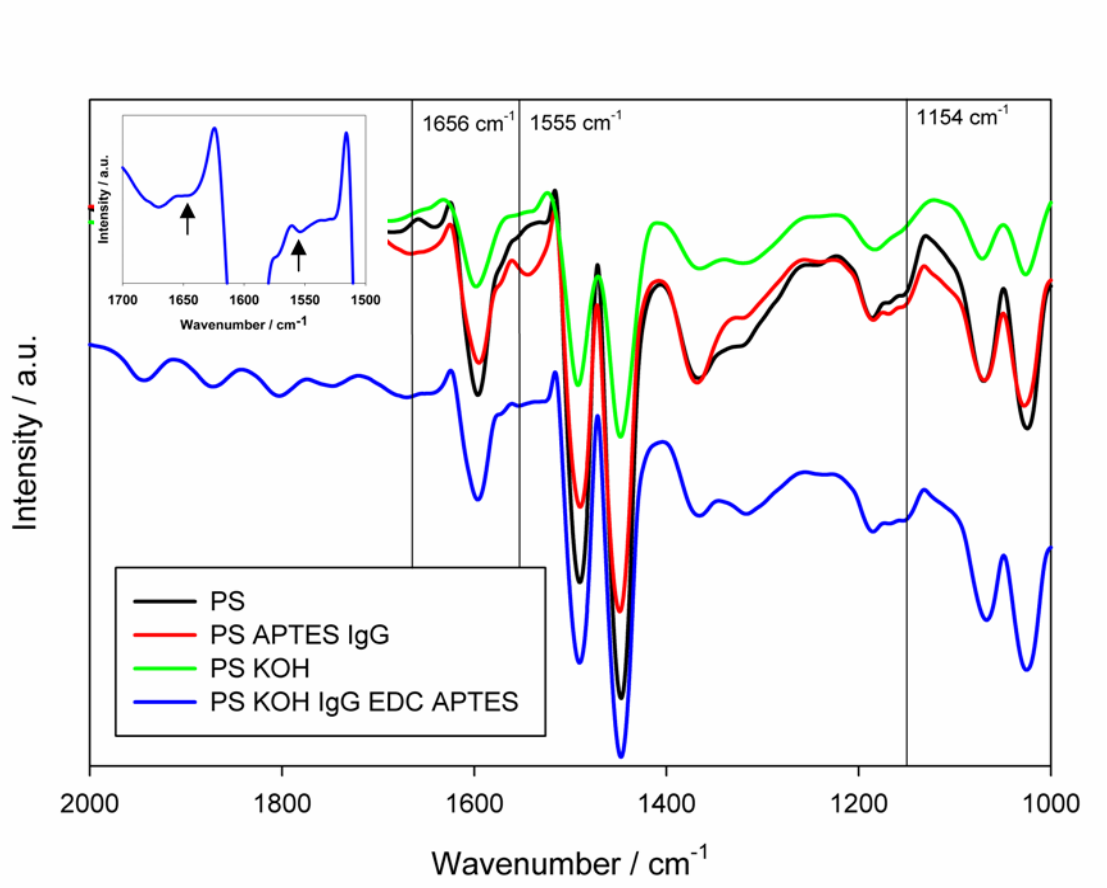


Figure S3. FTIR spectra comparing antibody ionically bound and covalently bound to APTES and its adsorption onto PS. Inset: Amide bands for the EDC-assisted coupling antibody to APTES.

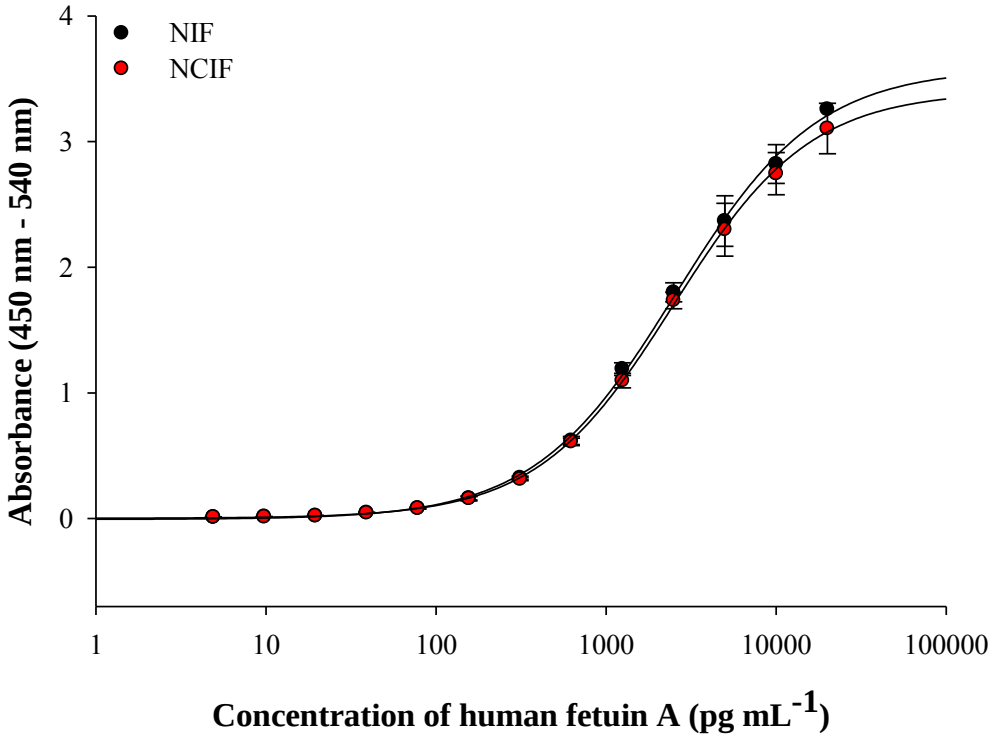


Figure S4. Comparison of the NIF- with NCIF-based sandwich ELISA. All experiments were done in triplicate with the error bars representing standard deviations**.**

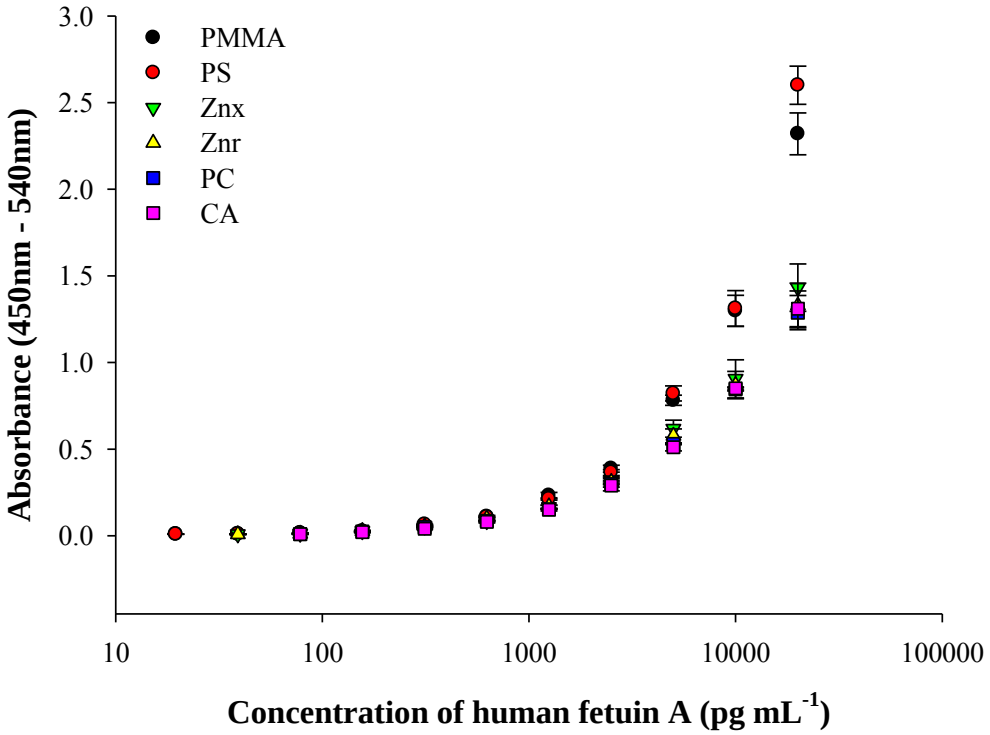


Figure S5. Demonstration of the multisubstrate-compatibility of the developed sandwich ELISA procedure. The assay was performed on various commercially-relevant substrates, i.e. polystyrene (PS), poly(methyl methacrylate) (PMMA), Zeonex™ (Znx), Zeonor™ (Znr), polycarbonate (PC) and cellulose acetate (CA), using our microtiter plate format[1,2]**.** All experiments were done in triplicate, while the error bars represent standard deviation.

Figure S6 shows the FTIR spectra of PS+APTES+IgG beads immersed in 0.5% APTES aqueous solution over 1 and 4 weeks to determine the stability of the materials. No major changes in the FTIR bands associated with IgG were observed, however, the Si-O-Si band centered at 1154 cm^{-1} has grown substantially, indicating the polymerization of the APTES in water during the course of the four weeks.

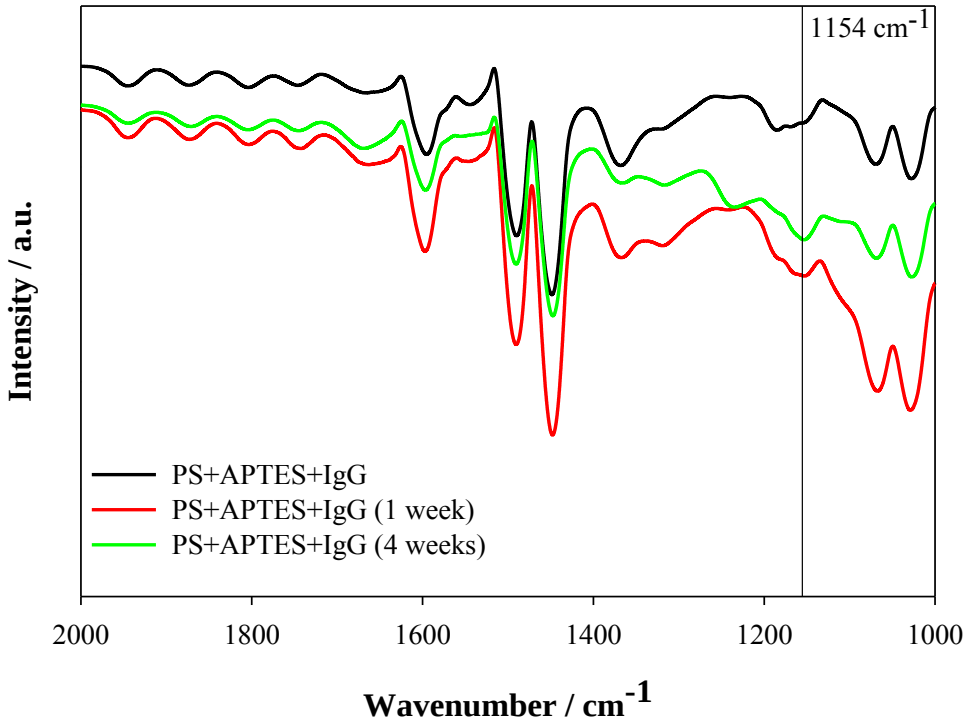


Figure S6. FTIR spectra of IgG-bound PS surface immersed in 0.5% (v/v) APTES solution for 1 and 4 weeks.

Table S1. Comparative analysis, based on the duration of various sandwich ELISA steps, of the NIF-, CovIF- and CIF-based sandwich ELISAs for the detection of HFA

Immunoassay Steps	**NIF-based sandwich ELISA**	**CovIF-based sandwich ELISA**	**CIF-based sandwich ELISA**
Immobilization of mouse anti-HFA	30 min at RT	2 h 20 min at RT and 37^{o} C	Overnight
Blocking buffer (BSA)	30 min	30 min	2 h
Recombinant HFA	1 h	1 h	2 h
Binding of biotinylated goat anti-HFA	1 h	1 h	2 h
Binding of Streptavidin-HRP	20 min	20 min	20 min
TMB substrate assay	20 min	20 min	20 min
Total immunoassay duration	~ 4 h	~ 6 h	~ 20 h

References

1. Dixit, C. K., Vashist, S. K., MacCraith, B. D. & O'Kennedy, R. Multisubstrate-compatible ELISA procedures for rapid and high-sensitivity immunoassays. *Nat. Protoc*. **6**, 439-445 (2011).
2. Vashist, S.K. *et. al*. A multi-well plate for biological assays. WO Patent 2,010,044,083. (2010).