3-Aminopropyltriethoxysilane-Based Bioanalytical Procedures for Potential *In Vitro* Diagnostics

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During the last three decades, there have been rapidly growing use of 3-aminopropyltriethoxysilane (APTES) in bioanalytical procedures for the development of potential *in vitro* diagnostics (IVD) [1]. APTES plays a prominent role in the surface modification of bioanalytical platforms [2-10] and the immobilization of biomolecules [11-13]. It is a colorless liquid having a density of 0.946 g mL^{-1} at 25°C, and melting and boiling points of -70°C and 217°C, respectively. It has been widely used for the development of IVD, especially those based on enzyme-linked immunosorbent assays (ELISA), microgravimetry, surface plasmon resonance (SPR), surface acoustic wave (SAW) and nanomaterials [1]. This manuscript provides an overview of our developed APTES-based bioanalytical procedures and their immense potential for the development of critically-improved IVD.

The surface modification of bioanalytical platforms by APTES is the preliminary step for the development of prospective IVD applications. This can be accomplished by the generation of hydroxyl groups on the surface by treatment with KOH/NaOH, plasma treatment, piranha solution or acids, followed by incubation with APTES for a few hours at room or elevated temperatures. Chemical vapor deposition (CVD) is another most widely used method for the deposition of APTES on the surface. Several prospective immobilization strategies have been developed for the binding of antibodies, enzymes and other biomolecules to the surfaces of diverse APTES-functionalized bioanalytical platforms [1]. These include covalent, oriented, covalent-oriented and site-specific approaches apart from strategies based on the use of affinity tags, peptide nucleic acid (PNA) and deoxyribonucleic acid (DNA). The extensive details of various silanization and biomolecular immobilization procedures are provided elsewhere [1].

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We have developed a wide range of IVD procedures based on enzyme linked immunosorbent assay (ELISA) and surface plasmon resonance, where APTES was employed as a surface modification and/or antibody immobilization agent. Our initial IVD procedure was based on sandwich ELISA [2, 5] (Figure **1**), which involved a lengthy procedure for the silanization of polystyrene microtiter plate (MTP). It involved the pretreatment steps of ethanol, KOH and O_2 plasma in order to generate the desired hydroxyl groups for the subsequent binding of APTES molecules. The MTP's surface was then functionalized with APTES by incubating for 1 h at 80°C in a vacuum desiccator. Thereafter, the capture antibodies were bound to the silanized MTP by heterobifunctional crosslinking using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and sulfo-N-hydroxysuccinimide (sulfoNHS). The subsequent sandwich ELISA steps were similar to that of the conventional sandwich ELISA procedure being used in the commercial IVD kit. The developed IVD procedure was multisubstrate-compatible when employed with our modified MTP format [14] and was significantly better than the commercial ELISA. It enabled the detection of human fetuin A (HFA) with a linear range of 9 pg mL^{-1} to 20 ng mL^{-1} and sensitivity of 39 pg mL^{-1}, which was 16-fold more sensitive and 3-fold more rapid than conventional ELISA. The developed antibody immobilization procedure was further improved by obviating the use of ethanol and O_2 plasma for silanization, and employing EDC alone for crosslinking [3, 4, 6, 7]. It led to the development of critically improved sandwich ELISA-based IVD procedures for the detection of HFA [7], human albumin (HA) [3] and human lipocalin-2 (LCN2) [4].

We then developed a signal enhanced immunoassay for LCN2 [15], where graphene nano platelets (GNPs) diluted in APTES were bound to the MTP's surface. Therefore, the surface modification of GNPs and MTP with APTES occurred together with the binding of GNPs to the MTP, thereby obviating any increase in immunoassay duration or complexity. The

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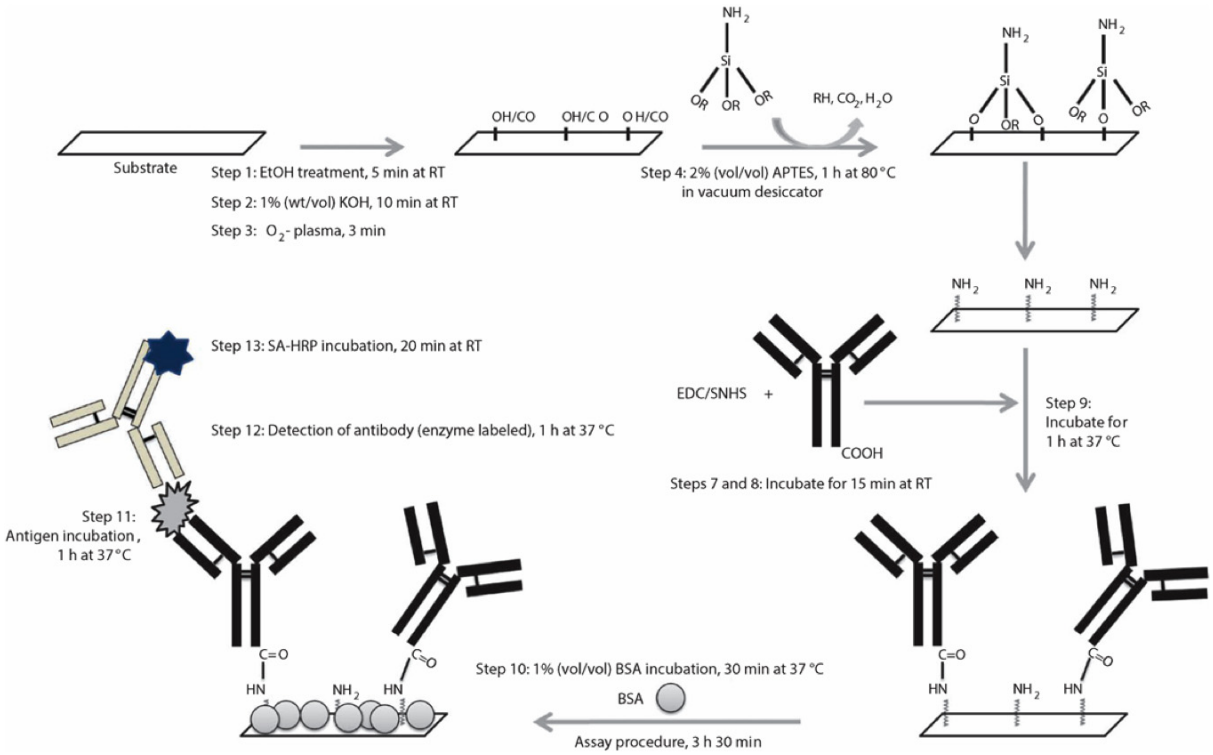

Figure 1: Highly-sensitive sandwich ELISA for the detection of human fetuin A (HFA). Reproduced with permission from Nature America Inc. [5].

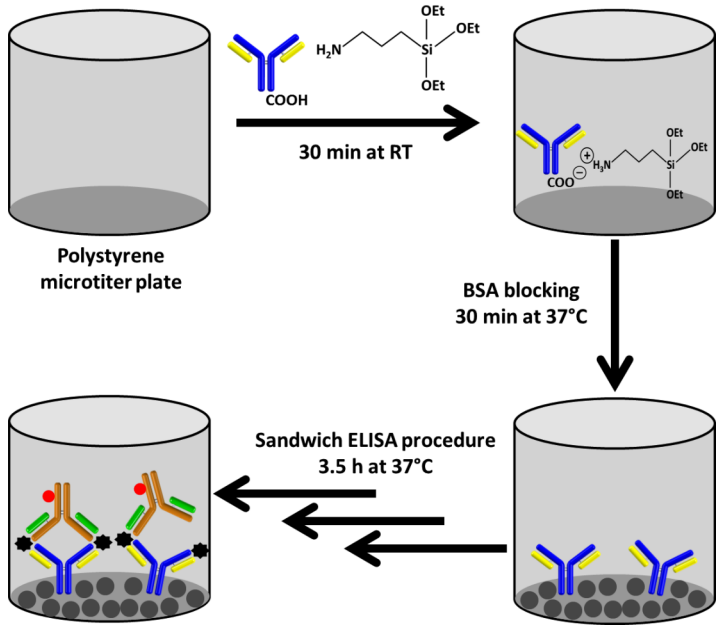

Figure 2: One-step antibody immobilization-based highly-sensitive sandwich ELISA for potential IVD. Reproduced with permission from Nature Publishing Group [11].

developed GNPs-based sandwich ELISA detected LCN2 in the linear range of 80-2560 pg mL^{-1} with the limit of detection of 0.7 pg mL^{-1}. It has 80-fold higher sensitivity and 3-fold lesser immunoassay duration than the commercial IVD kit. Moreover, it detected LCN2 in plasma, serum and whole blood without any interference from physiological substances.

Subsequently, we developed a novel and highly-simplified one-step antibody immobilization strategy [11] (Figure **2**), where antibody was diluted in APTES and bound to the polystyrene MTP's surface by incubating for 30 min. The antibody binds to APTES *via* ionic and hydrophobic interactions, which resulted in the formation of a stable complex. The developed one-

step antibody immobilization strategy was employed for the development of sandwich ELISA-based IVD for HFA, C-reactive protein (CRP), HA and LCN2. It detected HFA in the range of 4.9 pg mL^{-1}-20 ng mL^{-1} with LOD of 7 pg mL^{-1} and has 51-fold increased sensitivity than the commercial IVD kit. It has high analytical precision similar to that of commercial IVD kit and enables analyte detection in real sample matrices. The developed immunoassays were much better than the conventional immunoassays for all the tested biomarkers.

Lastly, we have recently developed a rapid one-step kinetics-based sandwich ELISA procedure for IVD [12] (Figure **3**), which enables the detection of HFA in about 30 min. The procedure involved a preliminary step for the preparation of leach-proof antibody-bound MTP, where the one-step antibody immobilization procedure is employed using EDC-activated antibody diluted in APTES (Figure **3A**). The capture antibody-bound MTP is then incubated with the entire sandwich ELISA components and the analyte solution for 15 min, which leads to the formation of sandwich immune complex by

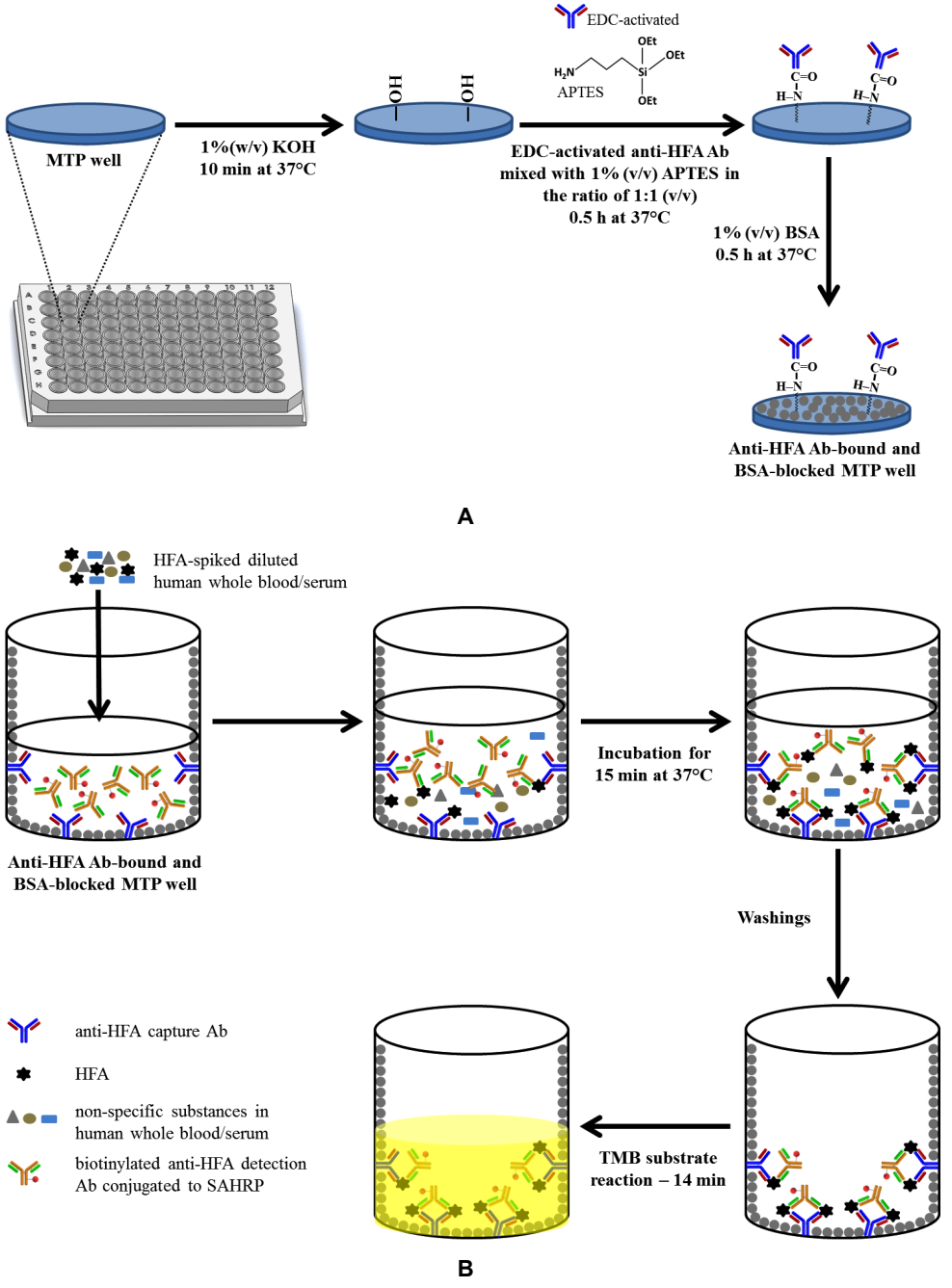

Figure 3: One-step kinetics-based sandwich ELISA for the detection of HFA in about 30 min. (**A**) Antibody immobilization procedure. (**B**) One-step kinetics-based sandwich ELISA procedure. Reproduced with permission from Elsevier B.V. [12].

one-step kinetics (Figure **3B**). Finally, the absorbance is measured after the enzyme-substrate reaction. The developed procedure was superior, rapid and simpler in comparison to our previously developed one-step kinetics-based immunoassay procedure based on the use of magnetic beads [16]. It detects HFA in the range of 0.1-243 ng mL^{-1} with LOD of 0.3 ng mL^{-1}. Taking into account the rapid immunoassay duration, the one-step kinetics-based IVD procedure have immense potential for the development of prospective point-of-care (POC) IVD kits. It is being used by us intensively for the development of smartphone-based colorimetric immunoassays using our smartphone-based colorimetric reader [17]. The procedure has high precision and detects HFA in whole blood and serum without any interference with physiological substances.

The APTES-based approaches have been further employed for the development of SPR-based label-free and real-time immunoassays. Our initial SPR immunoassay procedure was based on a lengthy procedure for the preparation of antibody-bound SPR chip, which involved surface cleaning, APTES-functionalization and heterobifunctional crosslinking of antibodies using EDC and/or sulfoNHS [8]. It detected HFA in the range of 0.6-20 ng mL^{-1} with sensitivity higher than that using commercial carboxymethyl-dextran (CM5-dextran) chip. Subsequently, we recently developed a rapid SPR immunoassay procedure that was analytically superior than our initial procedure as it employs our one-step antibody immobilization strategy for the rapid binding of capture antibodies [13] (Figure **4**). It has higher sensitivity than our initial procedure and can detect 0.3-20 ng mL^{-1} of HFA with LOD of 0.7 ng mL^{-1}.

We have also developed several mediatorless electrochemical biosensing schemes, using APTES-based strategies for surface modification and enzyme immobilization, for the detection of glucose in whole blood. In one scheme, glucose oxidase (GOx) was bound covalently to graphene-functionalized glassy carbon electrode (GCE) by EDC-based crosslinking [10]. The developed biosensor detected 0.5-32 mM glucose, thereby covering the complete pathophysiological range of glucose as desired for diabetic glucose monitoring. There was no interference from endogenous electroactive substances and drug metabolites. The developed electrodes demonstrated high production reproducibility and high storage stability. In another scheme, multiwalled carbon nanotubes (MWCNTs) were also employed in a similar manner and were observed to detect glucose in the range of 0.5-32 mM [9]. The effect of APTES concentration on the electrocatalysis of MWCNTs for glucose biosensing was also analyzed [18]. Moreover, a highly simplistic electrochemical biosensing scheme was devised, where GOx was drop-casted on a KOH-pretreated GCE followed by the drop-casting of APTES [19] (Figure **5**). The developed biosensor had a wide dynamic range as it detected 0.5-48 mM of glucose at -0.45 V. The developed procedure is the most rapid method for the preparation of glucose biosensor, which had excellent production reproducibility and high storage stability. In another scheme, a bienzyme mediatorless electrochemical biosensing strategy

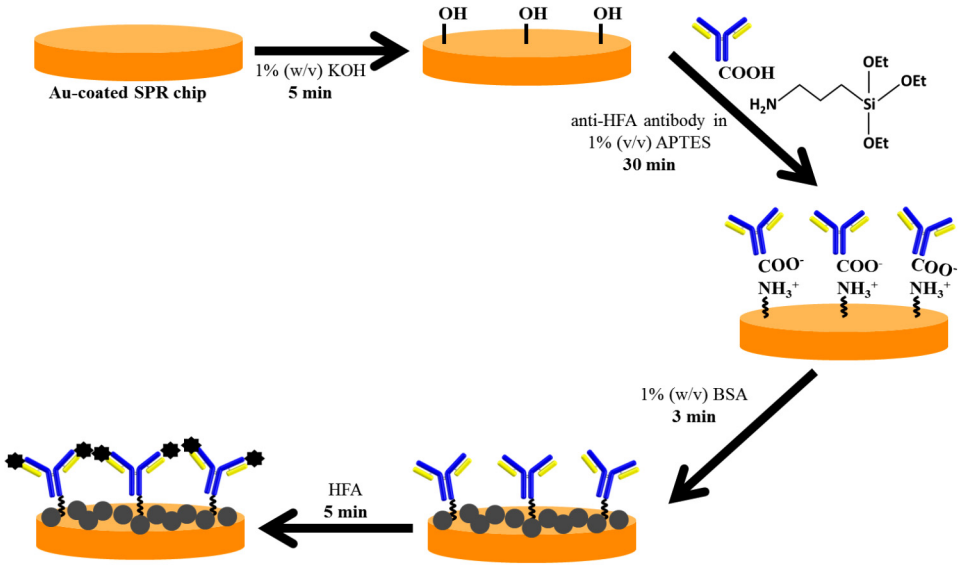

Figure 4: One-step antibody immobilization-based rapid SPR immunoassay for HFA. Reproduced with permission from the Royal Society of Chemistry [13].

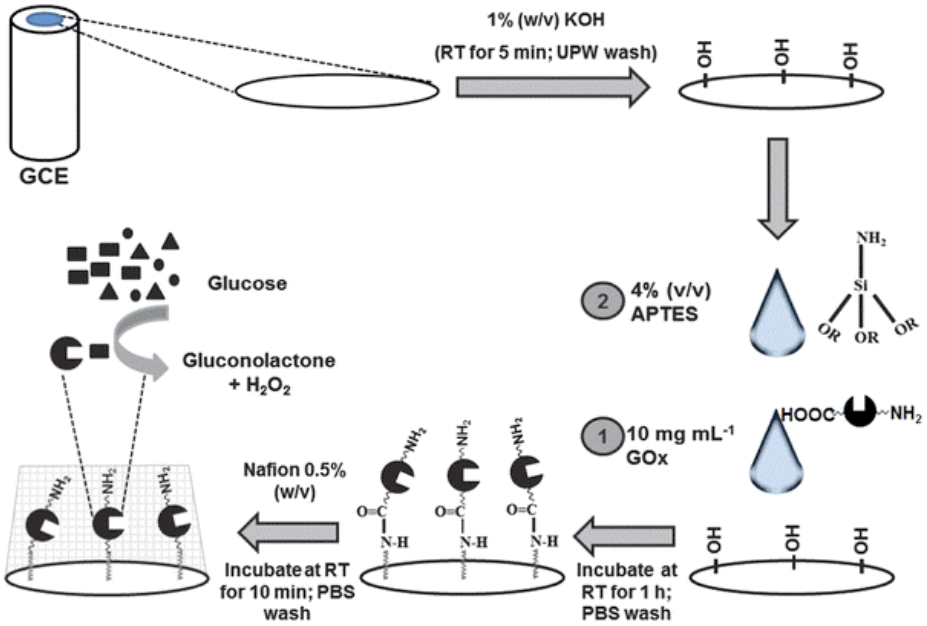

Figure 5: Bioanalytical procedure for the development of a highly-simplified mediatorless electrochemical glucose biosensor. Reproduced with permission from the Royal Society of Chemistry [19].

based on the use of GOx and horseradish peroxidase on GNPs-functionalized GCE was employed for the detection of glucose in whole blood [20]. It detected 0.5-64 mM of glucose at -0.45 V. These strategies have got immense potential as they can lead to the development of precise, robust and reliable blood glucose monitoring technologies [21-23], which is an essential requirement for more effective diabetic monitoring and management.

APTES has been widely employed as a surface modification agent and/or a dilution agent for biomolecular immobilization [1]. It binds to the surface of bioanalytical platforms in a leach-proof manner and provides the desired amino functional groups for the subsequent binding of antibodies, enzymes or other biomolecules. This is further complemented by the development of signal enhancement strategies, where nanomaterials diluted in APTES have been used for the immobilization of biomolecules. Our findings show that APTES-based bioanalytical procedures have led to considerable improvements in IVD in comparison to the conventional procedures. These continuous advances in the development of prospective APTES-based procedures are paving way to the next-generation of highly-simplified, cost-effective, rapid and ultrasensitive IVD.

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