

Rapid Immunodiagnostic Kits based on proprietary 1-step chemistry for covalent and leach-proof antibody immobilisation

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Abstract

We have developed a novel chemistry for the 1-step immobilization of antibodies on solid-substrate bioanalytical platforms. It enables the development of rapid immunodiagnostic (ID) kits that are analytically superior to the commercial ID kits using conventional antibody immobilization procedure. The developed chemistry is low-cost as it simply involves the dilution of capture antibodies in a particular concentration of 3-aminopropyltriethoxysilane (APTES), which is in fact cheaper than the phosphate buffered saline. It enables the leach-proof covalent binding of capture antibodies in just 30 min, which is >20-fold and >4-fold more rapid than the conventional (used in commercial ID kits) and our previously developed immobilization procedures, respectively. The immobilization chemistry was employed for the development of rapid ID kit, based on sandwich enzyme-linked immunosorbent immunoassay (ELISA), to detect human C-reactive protein (hCRP) in the dynamic range of 15.6-4000 pg/mL. The developed ID was very highly-sensitive and can detect the entire pathophysiological range of CRP in human serum after appropriate sample dilution. The developed chemistry has very high commercial potential as it will enable the development of very highly-sensitive ID kits for clinical, industrial and bioanalytical applications.

1 Motivation

Enzyme linked immunosorbent assay (ELISA) has been the gold standard for analyte detection in clinical, industrial, environmental and other bioanalytical settings. During the last two decades, there have been several advances in antibody immobilization chemistries [1-6] in order to improve the analytical sensitivity and decrease the overall immunoassay (IA) duration. However, most of these chemistries employ many steps or additional chemicals (such as crosslinking agents), which make the immobilization of antibodies on the solid substrate platforms a complex and costly procedure. Therefore, we developed a novel one-step antibody immobilization strategy [1], where the capture antibodies, diluted in a particular concentration of 3-aminopropyltriethoxysilane (APTES), were immobilized on the KOH-treated solid substrate platform in a leach-proof fashion in just 30 min. It was employed for the development of rapid immunodiagnostic (ID) kit for human C-reactive protein (hCRP), which had remarkably reduced IA duration i.e. >20-fold and >4-fold lesser than the conventional (used in commercial ID kits) and our previously developed immobilization chemistries [2-5], respectively, in addition to much better analytical sensitivity. The developed chemistry will have extensive applications in the development of rapid and very highly-sensitive ID kits, based on various IA formats/platforms, for disease biomarkers and analytes.

2 Materials and Method

2.1 Materials

The phosphate buffered saline (Cat.# 18912-014; PBS, pH 7.4) were procured from Invitrogen, while Tween 20 and Nunc microwell 96 well polystyrene plate (Cat.# 12-565-311) were purchased from Carl Roth GmbH and Fisher Scientific, respectively. The human CRP Duoset kit's (DY1707) components, i.e. anti-human CRP capture antibody, recombinant human CRP and biotinylated anti-human CRP detection antibody, were procured from RnD Systems, USA. Potassium hydroxide (KOH), 3,3',5,5'-tetramethylbenzidine (TMB) substrate, stop solution, bovine-serum albumin (BSA) and streptavidin-conjugated horseradish peroxidase (SA-HRP) were bought from Sigma-Aldrich, Germany. 3-APTES was obtained from ABCR, while CRP-free human serum (Cat.# 8CFS) was purchased from HyTest Ltd., Finland. The autoclave was from Systec GmbH, Germany; while the MTP reader used was Perkin Elmer Wallac VICTOR 1420 Multilabel Counter. All buffers and solutions were prepared in autoclaved ultrapure water – DNase and RNase free (Cat. # 10977; Gibco, Germany). The binding and washing buffers employed for the developed CRP IA were PBS with 0.1% BSA and PBS with 0.05% Tween 20, respectively. The working aliquots of commercial lyophilized human CRP were made in 20 mM Tris-HCl, pH 8.0 with 0.1% BSA (as mentioned in the product brochure), while the CRP spiking was done in diluted human serum (diluted in the binding buffer). In the developed hCRP IA, the washings with DIW and washing buffer were done with 300

μL of the respective solutions, while 100 μL was taken for each of the various solutions i.e. KOH, anti-hCRP capture antibody (diluted in APTES), hCRP, biotinylated anti-hCRP detection antibody, HRP-conjugated streptavidin, and TMB substrate. The volume of the stop solution used was 50 μL .

2.2 Leach-proof covalent binding of capture antibodies

The 96-well MTP was treated with 1.0% (w/v) KOH for 10 min followed by five DIW washes. The capture anti-hCRP antibody (5 $\mu\text{g}/\text{mL}$; diluted 1:1 (v/v) in 1% APTES) was then dispensed to each of the desired MTP's wells and incubated for 30 min at room temperature (RT) inside the fume cabinet. The anti-hCRP antibody-bound MTP was subsequently washed five times with washing buffer in order to remove excess unbound antibodies from the surface.

2.3 Developed hCRP IA procedure

The anti-hCRP antibody-bound MTP wells were blocked with 5% (v/v) BSA (diluted in 0.1M PBS, pH 7.4) for 30 min at RT and subsequently washed five times with washing buffer. The antibody-coated MTP wells were then incubated with hCRP (varying concentration; 15.6-4000 pg/mL) for 1 h at RT, and, subsequently washed five times with washing buffer. Thereafter, biotinylated anti-HFA detection antibody (0.17 $\mu\text{g}/\text{mL}$) was provided to all MTP wells and incubated for 1 h at RT followed by five washings with washing buffer. Subsequently, HRP-conjugated streptavidin, at a dilution of 1:3000, was added to all MTP wells and incubated for 20 min at RT followed by five washings with washing buffer. The detection anti-hCRP bound-MTP was then incubated with TMB substrate for 17 min at RT. Finally, the enzyme-substrate reaction was stopped by adding the stop solution. The absorbance of the solution was measured at 450 nm taking 570 nm as the reference wavelength. All the experiments were carried out in triplicate. The control for this study was zero ng/mL HFA in PBS, whose signal was subtracted from all assay values. Various experimental controls were additionally employed to determine the efficiency of BSA blocking and its interaction with various IA components, such as hCRP, biotinylated anti-hCRP antibody and SA-HRP. All datasets were subjected to standard curve analysis using SigmaPlot software, version 11.2. The commercial hCRP ELISA was performed exactly as per the instructions provided by the kit manufacturer.

3 Results and Discussion

The developed 1-step antibody immobilization chemistry enables the covalent and leach-proof binding of capture antibodies on the solid-substrate bioanalytical platforms, which leads to critically improved ID kits. The chemistry was employed for the development of sandwich ELISA-based rapid ID kit for hCRP, which needs to be precisely determined in neonatal sepsis, cardiovascular diseases,

meningitis and infectious/inflammatory conditions. The developed ID kit was rapid, highly-sensitive, analytically superior and cost-effective [1] than the commercial ID kit (RnD Systems, USA) and our previously developed ID procedures [2-5]. The 1-step antibody immobilization procedure is multisubstrate-compatible and leads to the leach-proof binding of antibodies to the solid substrate (Fig. 1A). It involves just 30 min incubation of antibody solution [anti-hCRP capture antibody and 1% APTES mixed 1:1 (v/v)] on KOH-pretreated MTP (Fig. 1A). The subsequent process steps are similar to those of the commercial kit. The devised strategy employs APTES both as dilution agent for antibodies and as surface functionalization agent for MTP. The developed ID kit has significantly reduced the sandwich immunoassay duration by more than 4-fold, i.e. from 19 h (in the commercial kit) to \sim 4 hours with better analytical performance. It has dynamic range of 15.6-4000 pg/mL with linearity between 125-2000 pg/mL (Fig. 1B), which can detect the entire pathological range of human CRP in serum (0.08-480 $\mu\text{g}/\text{mL}$) after appropriate sample dilution. The limit of detection, half-maximal effective concentration, inter-day and intra-day variability were 28 pg/mL , 827 pg/mL , 1.2-10.1%, 0.1-8.9%, respectively.

The developed kit detected CRP in buffer and human serum without any interference from involved process steps and CRP IA components (Fig. 1C). 0.5% (v/v) APTES was found to be the optimized final concentration (Fig. 1D), while 0.5 h was the optimized duration for 1-step antibody immobilization (Fig. 1E). The developed kit precisely determines the CRP concentration as it had perfect correlation with the commercial kit with Pearson's correlation coefficient of 1. The stability of the capture anti-hCRP antibody-bound MTP plates is in progress, which are expected to have prolonged shelf-life taking into account the leach-proof antibody immobilization.

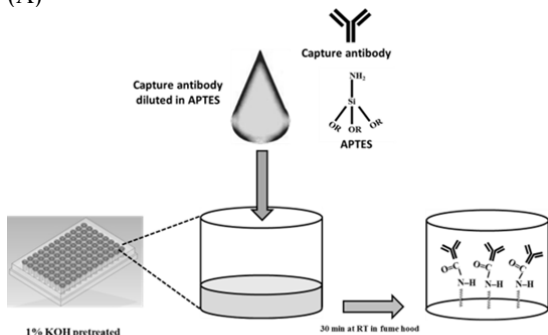
The developed 1-step antibody immobilization chemistry has been successfully demonstrated for the detection of various disease biomarkers in complex sample matrices i.e. human whole blood, plasma and serum. It enabled the development of critically improved ID kits in all the IAs that we have performed till date.

The 1-step antibody immobilization chemistry was further used for the development of rapid surface plasmon resonance (SPR)-based real-time and label-free microfluidic IAs for hCRP and many other biomarkers in just 10 min. The developed capture antibody-bound SPR chip did not show any decrease in analyte detection even after 3 months of storage and multiple reuse, which clearly demonstrate the leach-proof nature of antibody immobilization. Moreover, the developed SPR IA format based on our chemistry was the most rapid and highly-sensitive in comparison to our previously developed and the commercial SPR chips-based IA formats [6]. The immobilization chemistry was further employed for our next-generation of instrument-free and low-cost smartphone-based ID kits using our recently developed proprietary smartphone-based colorimetric readers (costing $<€$ 100 prototype) that

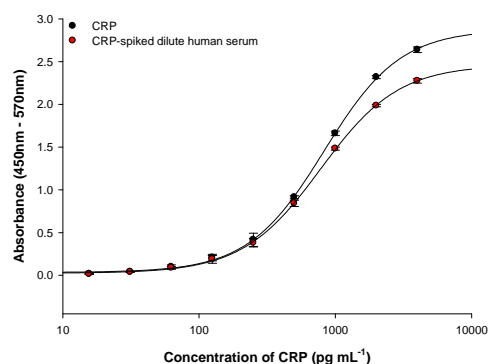
have higher sensitivity than MTP readers (costing €10,000-40,000) [7].

We are actively looking for clinical, industrial and bio-analytical partners to employ our developed rapid and cost-effective immunodiagnostic procedure for potential end-user applications. We intend to use the developed chemistry to manufacture highly prospective and commercially-viable ID kits, in various IA formats, for various disease biomarkers/analytes that target the mass-market of *in vitro* diagnostic kits.

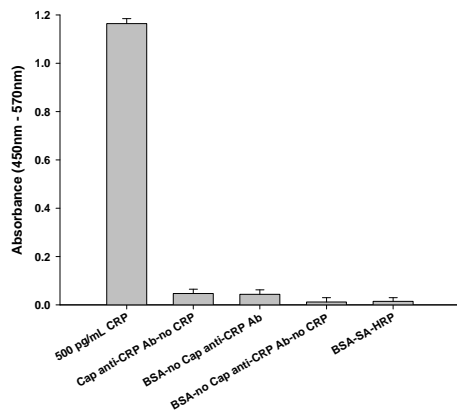
(A)



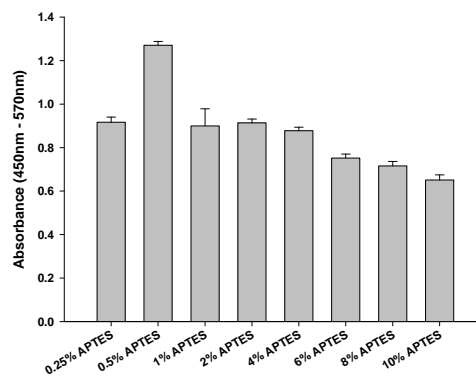
(B)



(C)



(D)



(E)

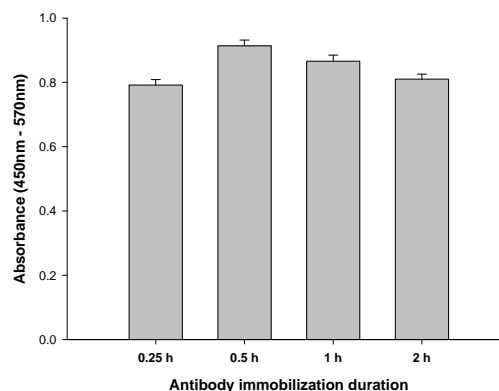


Figure 1 Developed Rapid CRP immunodiagnostic Kit. (A) Schematic of the novel 1-step antibody immobilization chemistry, (B) Detection of CRP in PBS buffer (with 0.1% BSA) and CRP-spiked dilute human serum, (C) Experimental process controls, (D) Optimization of final APTES concentration (after mixing 1:1 (v/v) with antibody), and (E) Optimization of antibody immobilization duration.

4 Conclusion

The developed 1-step antibody immobilization chemistry enables the leach-proof covalent immobilization of capture antibodies on the solid substrates in just 30 min. It leads to very highly-sensitive and cost-effective ID kits with significantly reduced overall IA duration and superior analytical performance than commercial and our previously developed ID procedures. The chemistry was used to develop hCRP ID kit, which detects hCRP in the dynamic range of 15.6-4000 pg/mL with the LOD of 28 pg/mL. The developed hCRP ID kit correlated perfectly with the commercial kit and can detect hCRP in human serum. The chemistry has very high commercial potential as it can be employed for a wide-range of bioanalytical applications.

5 Literature

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