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One-step kinetics-based immunoassay for the highly sensitive detection of C-reactive protein in less than 30 min

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ABSTRACT

This article reveals a rapid sandwich enzyme-linked immunosorbent assay (ELISA) for the highly sensitive detection of human C-reactive protein (CRP) in less than 30 min. It employs a one-step kinetics-based highly simplified and cost-effective sandwich ELISA procedure with minimal process steps. The procedure involves the formation of a sandwich immune complex on capture anti-human CRP antibody-bound Dynabeads in 15 min, followed by two magnet-assisted washings and one enzymatic reaction. The developed sandwich ELISA detects CRP in the dynamic range of 0.3 to 81 ng ml^{-1} with a limit of detection of 0.4 ng ml^{-1} and an analytical sensitivity of 0.7 ng ml^{-1}. It detects CRP spiked in diluted human whole blood and serum with high analytical precision, as confirmed by conventional sandwich ELISA. Moreover, the results of the developed ELISA for the determination of CRP in the ethylenediaminetetraacetic acid plasma samples of patients are in good agreement with those obtained by the conventional ELISA. The developed immunoassay has immense potential for the development of rapid and cost-effective in vitro diagnostic kits.

Episodal or chronic inflammation is a major risk factor for cardiovascular diseases and death in the Western world. It is likely multifactorial and includes chronic infections, pro-inflammation by chemicals and nutrition, physical and psychosocial stress, and the formation of advanced glycation end-products. C-reactive protein (CRP)[1] is produced by hepatocytes in response to a variety of inflammatory cytokines such as interleukin (IL)-6, IL-1, and tumor necrosis factor alpha (TNFα), which are released during infection or tissue inflammation. It results in continuous oxidative stress-related vascular injury, as evidenced by increased plasma concentrations for acute-phase proteins (CRP and serum amyloid A) and specific cytokines (IL-6 and TNFα) [1]. CRP, a pentameric protein with a molecular weight of 118 kDa, is a member of a class of acute-phase reactants that mediates innate and adaptive immunity [2–4]. It binds to phosphocholine and related molecules on microorganisms and plays an important role in the host defense. As an early indicator of infectious or inflammatory conditions [5,6], it is usually elevated in patients with neonatal sepsis (affecting 1–10 per 1000 live births) [7–9], meningitis, pancreatitis, pneumonia, pelvic inflammatory disease, and occult bacteremia. Significantly elevated serum CRP levels are associated with malignant diseases, bacterial infections, and very high 30-day mortality rates in hospitalized medical patients [10]. The monomeric form of CRP has been demonstrated to bind to the surface of damaged cells and platelets and is able to activate the complement cascade; thus, it plays an important role in promoting inflammation. Membrane binding to monocytes and macrophages occurs via binding thorough M-ficolin, resulting in a potent pro-inflammatory signaling cascade in tissues [11]. The American Heart Association, in conjunction with the Centers for Disease Control and Prevention, identifies CRP as the best inflammatory marker for clinical diagnosis [12].

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[1] *Abbreviations used:* CRP, C-reactive protein; IL, interleukin; TNFα, tumor necrosis factor alpha; hsCRP, high-sensitivity CRP; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; BSA, bovine serum albumin; Ab, antibody; SA–HRP, streptavidin-conjugated horseradish peroxidase; EDTA, ethylenediaminetetraacetic acid; MTP, microtiter plate; UPW, ultrapure water; LOD, limit of detection; OD, optical density.

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CRP has been proven as the strongest independent predictor of cardiovascular events [13–19] such as heart attacks, ischemic stroke, coronary artery disease, and acute myocardial infarction. It has also been considered as an independent predictor for the development of diabetes in men [20] and a marker for atherosclerotic cardiovascular risk [21]. CRP levels are important indicators of cardiac tolerance associated with cardiorespiratory fitness [14]. Elevated high-sensitivity CRP (hsCRP) correlates with the presence of insulin resistance and type 2 diabetes mellitus [22] and is associated with many features of metabolic syndrome [23]. hsCRP levels and other markers may predict the development of dementia, including that in Alzheimer's disease [19]. They can predict long-term cardiovascular risk in individuals without any prior evidence of cardiovascular disease [17]. The technical achievement of repeated CRP measurements in an acute setting provides clinicians with valuable information to establish the correct disease diagnosis and circumvent the unnecessary use of antibiotics.

CRP levels in normal human serum are usually less than 10 μg ml^{-1} [3]. The median physiological serum concentration of CRP in humans is 0.8 μg ml^{-1}, which can reach up to 350 to 400 μg ml^{-1} in several disease states. CRP levels are in the ranges of 10 to 40, 40 to 200, and more than 200 μg ml^{-1} in mild inflammation and viral infections, active inflammation and bacterial infections, and severe bacterial infections and burns, respectively [3]. CRP levels above the cutoff point of 3 μg ml^{-1} are associated with an increased risk of occlusive arterial disease, especially acute coronary syndrome. The two different CRP concentration ranges, normal (0.2–480 μg ml^{-1}) and high sensitivity (0.08–80 μg ml^{-1}), need to be detected in neonatal sepsis [3]. CRP levels beyond the cutoff point of 5 μg ml^{-1} are indicative of neonatal sepsis. Initially, an hsCRP assay is performed. But if the CRP levels are more than 80 μg ml^{-1}, a normal CRP assay is also performed.

Enzyme-linked immunosorbent assay (ELISA) has always been the "gold standard" for the detection and quantification of CRP. However, various other CRP assay formats [4,24–44] have also been devised during the past decade such as those based on immunoturbidimetry [24,34], lateral flow [42], homogeneous bead-based immunoassay [45], piezoresistive cantilever-based immunoassay [33], surface plasmon resonance [36], impedimetry [46], chemiluminescent immunoassay [26], electrochemistry [39], microfluidics [30], and reflectometric interference spectroscopy [41].

Here we report a highly simplified and cost-effective one-step kinetics-based sandwich ELISA that enables the detection of hsCRP in real sample matrices in less than 30 min (Fig. 1). It has approximately 12-fold reduced immunoassay duration, 2.5-fold reduced analysis cost, and a critically reduced number of process steps. Indeed, it requires only 2 washing steps in comparison with 24 washing steps in the conventional sandwich ELISA. Therefore, it is a potential rapid immunoassay format that can be reliably employed in health care, industrial, and bioanalytical settings for precise analyte detection.

Materials and methods

Materials

Phosphate-buffered saline (PBS, 0.1 M, pH 7.4), 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit, and bovine serum albumin (BSA) were procured from Thermo Scientific, whereas Dynabeads (M-280 tosylated, 2.8 μm diameter, 30 mg ml^{-1}) were procured from Invitrogen. The human CRP Duoset kit components—anti-human CRP capture antibody (Ab), recombinant human CRP, biotinylated anti-human CRP detection Ab, and streptavidin-conjugated horseradish peroxidase (SA–HRP)—were procured from R&D Systems (USA). Sulfuric acid, Tween 20, and Nunc microwell 96-well polystyrene plates (flat bottom [nontreated], sterile) were purchased from Sigma–Aldrich. Human whole blood (HQ-Chex level 2) and CRP-free human serum were procured from Streck (USA) and HyTest (Finland), respectively. The anonymized clinical samples (i.e., ethylenediaminetetraacetic acid [EDTA] plasma samples of patients) containing varied levels of CRP were provided by University Hospital Ulm (Germany). The magnetic holder (Quadermagnet) containing 24 magnets, with each magnet spaced in the

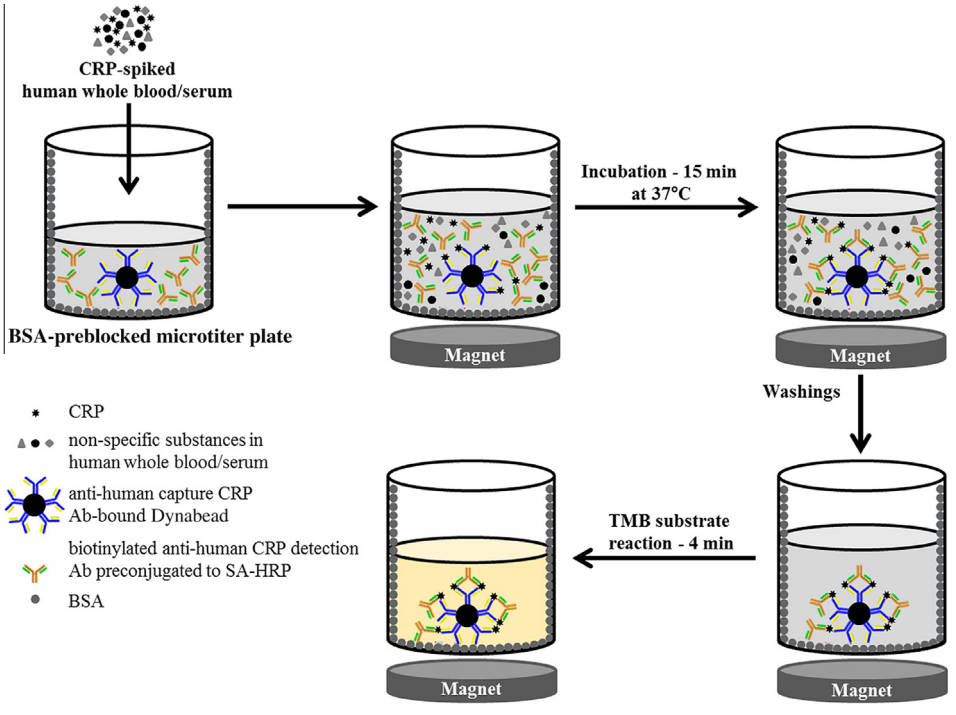

Fig.1. Schematic of the developed one-step kinetics-based sandwich ELISA for the detection of CRP.