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EDITORIAL

# Prospects and advancements in C-reactive protein detection

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## Abstract

C-reactive protein (CRP) is one of the earliest proteins that appear in the blood circulation in most systemic inflammatory conditions and this is the reason for its significance, even after identification of many organ specific inflammatory markers which appear relatively late during the course of disease. Earlier methods of CRP detection were based on the classical methods of antigen-antibody interaction through precipitation and agglutination reactions. Later on, CRP based enzymatic assays came into the picture which were further modified by integration of an antigen-antibody detection system with surface plasma spectroscopy. Then came the time for the development of electrochemical biosensors where nanomaterials were used to make a highly sensitive and portable detection system based on silicon nanowire, metal-oxide-semiconductor field-effect transistor/bipolar junction transistor, ZnS nanoparticle, aptamer, field emission transmitter, vertical flow immunoassay *etc*. This editorial attempts to summarize developments in the field of CRP detection, with a special emphasis on biosensor technology. This would help in translating the latest development in CRP detection in the clinical diagnosis of inflammatory conditions at an early onset of the diseases.



**Key words:** C-reactive protein; Inflammation; Diagnostic methods; Antibody; Biosensors

**Core tip:** Over time, C-reactive protein (CRP) has emerged as a versatile marker for the detection of systemic inflammatory conditions, providing preliminary information to clinicians for continuing with a more specific diagnostic methodology. Advancements in electroanalytical chemistry and knowledge of nanomaterials have helped modern age researchers to miniaturize detection systems with an enhanced level of specificity and sensitivity of CRP detection. Further research should be directed in this area to devise a better diagnostic platform that can detect the change in CRP level at a very early stage of the onset of inflammatory conditions.

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## INTRODUCTION

In humans, there are many acute phase proteins whose level in blood plasma increases or decreases in response to inflammation (acute phase reaction). Some of the acute phase proteins are C-reactive protein (CRP), mannose binding protein, complement factors, serum amyloid A, fibrinogen, retinal binding protein, ceruloplasmin and antithrombin. Amongst them, CRP is the most important, sensitive and systemic marker of inflammation identified in the human body as its level rises rapidly in the blood plasma in response to a large number of foreign bodies, infections, tissue damage, renal and cardiovascular diseases[1]. It is secreted by hepatocytes

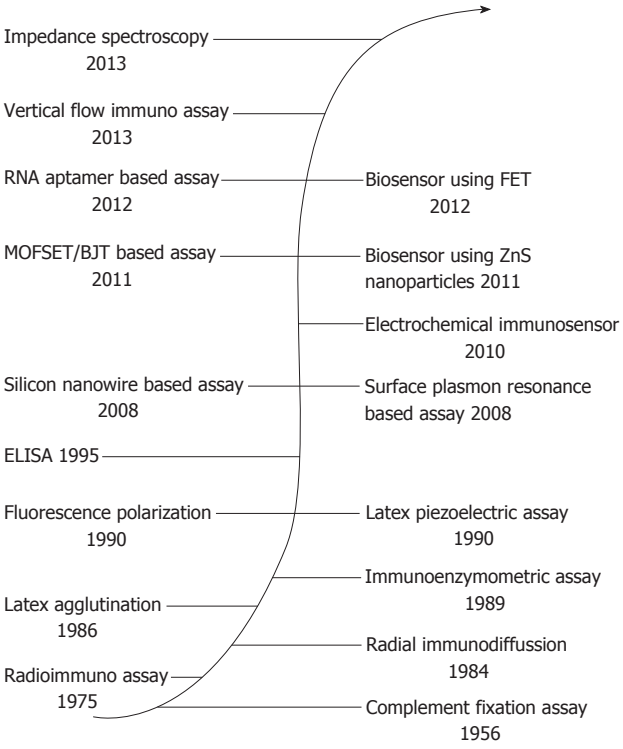


**Figure 1 Diagrammatic representation of the advancement in C-reactive protein detection.** MOFSET/BJT: Metal-oxide-semiconductor field-effect transistor/bipolar junction transistor; FET: Field effect transistor; ELISA: Enzyme-linked immunosorbent assay.

in response to cytokines, like interleukin 6, interleukin 1, tumor necrosis factor alpha *etc*[2]. CRP ($M_r$ 115,135), a member of the pentraxin family of calcium dependent ligand binding plasma protein, is composed of 5 non-glycosylated polypeptide subunits, each of which is composed of 206 amino acid residues. Polypeptide units associate with each other through non-covalent bonding in an annular configuration forming cyclic pentameric symmetry. The ligand binding site of CRP comprises of loops with two calcium ions. During inflammation, phosphocholine present on necrotic or apoptotic cells binds at the active site of CRP, thereby activating the classical complement pathway essential for opsonization and induction of pro-inflammatory pathophysiological effects. Additionally, it activates the complement pathway but also increases a respiratory burst of neutrophils, encourages expression of adhesion molecules and synthesis of tissue factors. Based on this clinical importance of CRP, attempts have been made in this editorial to summarize the chronological development in the field of CRP detection. The physiological level of CRP in human plasma is 2 mg/L, whereas during inflammatory conditions, its concentration rises significantly in 6-8 h, even reaching up to 300 mg/L in the next 48 h. CRP level in patients with a cardiovascular disorder and/or myocardial infarction at the time of admission to the hospital have been observed to be above the physiological range (more than 3 mg/L)[3]. CRP deposits in the arterial walls during atherogenesis, thereby activating the complement pathway and augmenting the development of several cardiovascular disorders[4]. Abraham *et al*[5] observed a higher level of CRP (14.3 mg/L ± 11.2 mg/L) in patients before dialysis who were susceptible to chronic kidney disorder, renal failure or kidney malfunction. A higher concentration of CRP is also found during late pregnancy. People with obesity and high body mass index also have a higher level of CRP in blood plasma[6]. In a study by Lee *et al*[7], a raised level of high sensitivity CRP (hsCRP) was also correlated with the development of cancer. Hence, CRP is an important marker of clinical conditions like local and systemic inflammation, myocardial diseases, obesity *etc*. The prospect of developing a highly specific and sensitive method of detection of CRP at an early stage of these clinical conditions has been attempted by various research groups. The overall chronological development is elucidated in Figure 1.

Conventional methods of CRP detection rely on precipitation by C-polysaccharide of *Pneumococcus*, tube precipitation, complement fixation, latex agglutination, radioimmunoassay, radial immunodiffusion and fluorescence polarization. Detection of CRP by radial immunodiffusion uses radial immunodiffusion plates made of agarose containing 1% rabbit anti-human CRP. Sera samples are added into the wells punched on them and the diameter of the radial rings measured after a 48 h incubation period. The greater the diameter of the precipitation ring, the higher the CRP concentration in the serum. The time taken for the assay and its semi-quantitative nature are the major limitations of this detection system[8]. As an improvement of the previous technique, the latex agglutination method was developed which employs inert latex particles coated with anti-human CRP antibody. In the presence of CRP in the patient's serum, the agglutination reaction can be seen between anti-human CRP and CRP moieties. Unlike the precipitation reaction, it takes less time but still has the limitation of being semi-quantitative in nature[9]. In 1990, Kurosawa *et al*[10] developed a latex piezoelectric immunoassay using a piezoelectric quartz crystal which acts as the sensing element for the change in viscosity or density in the solution due to aggregation of latex particles. It negated the disadvantages of previous methods of detection of CRP using agglutination through the use of a latex bearing antibody with no film. Earlier piezoelectric assays employed the formation of an antibody coated thin film latex on a crystal by which the oscillating frequency of the crystal reduces. This approach removed the drawbacks of previous methods in terms of labeling reporter molecules and through improving the assay sensitivity. Furthermore, an immunoenzymometric assay for determination of CRP using two antibodies has been developed by Käpyaho *et al*[11]. It is a simple assay consisting of a single immunological reaction between CRP and peroxidase labeled antibody with another antibody attached to the wall of the test tube. The immune complex formed is determined by a colorimetric assay using a peroxidase substrate. The sensitivity of this technique is comparable to the turbidimetric method of CRP detection. However, concerns about enzyme stability, shelf life and time taken for detection raise the question of its practical applications and shelf

life of the diagnostic system[11]. An enzyme-linked immunosorbent assay (ELISA) kit for the detection of CRP (Cell Biolabs Inc., San Diego, CA, United States) has anti-CRP antibody coated onto the microtiter plate that reacts with the CRP antigens. An enzyme linked secondary antibody in the presence of specific substrate gives rise to a colorimetric reaction whose optical density can be measured to estimate the level of CRP. The detection limit of this is up to 0.1 ng/mL but high false positives due to non-specific binding limits the availability of this methodology. Other major disadvantages include the long detection time, lower sensitivity, low stability, cross reactivity with the serum proteins, lack of miniaturization and on-site analysis.

Thus, in recent years, various biosensor based detection systems have been attempted for quick, sensitive and on-site detection of CRP. A biosensor is an analytical device utilizing a biological reaction between receptor and target molecules, converting the biological response into readable and quantifiable signals using transducers[12-15]. Lee *et al*[16] developed a biosensor based on surface plasma resonance spectroscopy which involved measurement of molecular interactions at the gold/silver surface of the sensing element, thereby measuring reflectance of light with respect to the refractive index of the surface of biosensing element that changes when CRP molecular species react at the fabricated unit. This technique uses poly (3-(2-((N-succinimidyl)succinyloxy)ethyl)thiophene) (P3SET) which is a polythiophene with pendant N-hydroxysuccinimide (NHS) ester group as a biolinker between the anti-CRP (bioreceptor) and sensing surface. A self-assembled monolayer (SAM) of P3SET formed on the gold surface and anti-CRP was immobilized covalently. When CRP reacted with sensor, there was a shift in the refractive index of P3SET/anti-CRP due to the formation of P3SET/anti-CRP/CRP on the sensing surface and reflectance was deviated. Hence, the reaction between anti-CRP immobilized on gold surface and CRP can be monitored using surface plasma resonance with a high sensitivity[15].

With advancements in nanotechnology, nanobiosensors have become very popular in recent times. In this regard, Lee *et al*[7] attempted the silicon-nanowire based fabrication process which follows a top-down approach of fabrication using micro-machining technology. In a new study, Yuan *et al*[17] developed a method to adjust sensitivity using a gated lateral bipolar junction transistor (BJT) in the metal-oxide-semiconductor field-effect transistor-BJT hybrid mode which was fabricated using the complementary metal-oxide-semiconductor manufacturing system. $Si_3N_4$ was immobilized on the layer on gold which was then immobilized on a floating gate using an electron beam evaporator. A die chip consisting of gated lateral BJT was then embedded onto a printed circuit board which was further connected to the vertical collector, base and lateral collector, and emitter. Internal metal layers were also employed to enhance the rate of current flow. Monoclonal anti-CRP antibodies were linked to the gold layer using SAMs of 11-mercaptoundecanoic acid, N-Hydroxysuccinimide and N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride. On reaction with CRP species, capacitance between the liquid and floating gate changes is measured. This change in capacitance has been used to determine the concentration of CRP with high sensitivity and reliability. The advantages of such a system are the small size, ease of manufacturing, low noise, high transconductivity, good selectivity and reproducibility. It has also been claimed that the developed system can be used for other biomarkers by changing the corresponding antibody.

A biosensor integrated with a microfluidic device has been also developed for the detection of CRP. In a report, CRP along with other cardiac marker troponin c has been detected simultaneously using a microfluidic device. The device developed a chip that acted as a microreactor for the simultaneous detection of CRP and troponin c. Antibodies with bioconjugated CdTe and ZnSe were used in the system. These quantum dots release $Zn^{2+}$ and $Cd^{2+}$ ions that are detected by square-wave anodic stripping voltammetry to enable the quantification of the two biomarkers. This electrochemical immunosensor has a detection range of 0.5-200 μg/mL, with a detection limit of 307 attomole in 30 μL for CRP[18]. Another method of detection which uses $Zn^{2+}$ ions for the detection of CRP was established by Cowles *et al*[19] where ZnS nanoparticles were used to transduce the signal *via* fluorescence spectroscopy. In this detection system, mouse anti-CRP coated magnetic microbeads were used. On addition of the serum sample containing CRP, the immune complex binds to these beads to which biotinylated mouse anti-CRP will fix. Neutravidin conjugated with ZnS nanoparticles will attach to this complex and in the presence of Flouzin3, a zinc ion selective fluorescence dye, generate a fluorescence signal. The bioassay possesses a detection limit of 10 pmol which makes it a highly sensitive method to detect CRP. In addition, it is also non-toxic and a less expensive system to fabricate. Another biosensor based on nanomaterial for the detection of CRP level was developed by Qureshi *et al*[20]. The detection system requires the use of specific interaction between CRP and its corresponding RNA aptamer. These CRP specific RNA aptamers are immobilized on carbon nanotubes activated gold interdigitated electrodes of capacitors *via* a physical adsorption. The selective binding of RNA aptamers with CRP is determined by measuring the capacitance after competitive binding between complementary RNA and CRP in pure forms and co-mixtures. It is a label-free method of detection based on affinity separation of target molecules with a limit of detection ranging from 1-8 μmol/L. Although the detection limit is very low, this method has merit in terms of a label-free approach and simple approach for detection of CRP. Kim *et al*[21] recently developed a biosensor using a field effect transistor in which silicon binding protein (SBP) is linked to surface protein A to simplify the tedious method of fabrication of the monolayer. SBP, an artificial protein,

**Table 1 Various C-reactive protein detection techniques and their characteristics**

| No. | Technique employed | Features | Ref. |
|---|---|---|---|
| 1 | Radial Immunodiffusion | Qualitative analysis in less than 48 h | Harris *et al*[8], 1984 |
| 2 | Latex agglutination | Time taken less than 24 h; qualitative analysis | Senju *et al*[9], 1986 |
| 3 | Latex piezoelectric assay | Uses quartz crystal and latex bearing antibody; more sensitive than conventional methods; less time required. | Kurosawa *et al*[10], 1990 |
| 4 | Immunoenzymometric Immunoassay | Single immunological reaction; sensitive; results comparable to turbidimetric detection | Käpyaho *et al*[11], 1990 |
| 5 | Surface plasma resonance spectrophotometry | High sensitivity; on-site analysis; SAM usage | Kim *et al*[15], 2008 |
| 6 | Silicon nanowire based assays | Micro-machining technology; higher detection limit | Lee *et al*[16], 2008 |
| 7 | MOFSET/BJT based technique | High sensitivity, change in capacitance measurement; reliable; small size; ease of manufacturing; good selectivity; highly reproducible; high trans conductivity | Yuan *et al*[17], 2011 |
| 8 | Electrochemical Immunosensor | Detection by square wave stripping voltammetry; quantitative analysis of 2 biomarkers; reproducible | Zhou *et al*[18], 2010 |
| 9 | Nanotechnology using ZnS nanoparticles | Detection by fluorescence spectrophotometry; highly sensitive; non-toxic; low cast system; highly specific | Cowles *et al*[19], 2011 |
| 10 | RNA aptamer based technology | Uses Carbon nanotube's interdigitated electrodes of capacitors; highly selective | Qureshi *et al*[20], 2012 |
| 11 | Biosensor using FET | Involves SBP linked in protein A; point of care testing system; on-site analysis | Kim *et al*[21], 2013 |
| 12 | Vertical flow Immunoassay | One-step assay; time taken 2 min; most rapid; employs gold nanoparticles | Oh *et al*[22], 2013 |
| 13 | Electrochemical impedance spectroscopy | Most advanced technique; uses gold and diamond spray in fabrication; highly sensitive; reusable without sensitivity being lost; good detection limit | Bryan *et al*[23], 2013 |

FET: Field emission transmitter; MOFSET/BJT: Metal-oxide-semiconductor field-effect transistor/bipolar junction transistor; SAM: Self-assembled monolayer; SBP: Silicon binding protein.

can bind to the silicon surface with no bi-linker. A fabricated device is treated with hot piranha solution to maximize the affinity of SBP-protein A complex onto the sensing area. The SBP-protein A is then immobilized on the surface of sensing element and dipped into the solution containing anti-CRP. The anti-CRP is coated onto the fabrication unit where CRP forms the immune complex which is transduced in a detectable signal. This is the application of a biosensor point-of-care-testing system with a detection limit comparable to that of ELISA. Oh *et al*[22] has recently developed a one-step biosensor for hsCRP detection using a vertical flow immunoassay. It is composed of a sample pad, flow through films (FTH), conjugate pad and nitrocellulose membranes (onto which anti-hsCRP and secondary antibodies are immobilized below the holes) which are stacked upon one another. Anti-hsCRP conjugated with gold nanoparticles is encapsulated in the conjugate pad. This fabricated system detects hsCRP 0.01-10 μg/mL within 2 min and is the most rapid biosensor to date (Table 1).

Recently, an optimized biosensor for a label-free detection of CRP in a blood serum sample has been developed by Bryan *et al*[23], based on electrochemical impedance spectroscopy using gold electrodes. SAMs of polyethylene glycol (HS-$C_{11}$-$(EG)_3$-$OCH_2$-COOH) with the help of ethanol and nitrogen gas are made and dipped into piranha solution. NHS is used to activate the carboxylate group and monoclonal anti-CRP is linked to monolayers covalently. This device detects CRP in blood on the basis of difference in impedance when CRP species reacts with the monoclonal anti-CRP antibody bound to SAM. This system of detection has a very good selectivity and reusability with no loss of apparent sensitivity. This can be considered one of the latest methods of CRP detection where no specific labeling is required *i.e.*, a label free detection system even through the picomolar detection limit.

## CONCLUSION

Our understanding of CRP detection systems has come a long way. Over the years, CRP has become a versatile inflammatory marker for the detection of systemic inflammatory conditions. In future, advancements in interdisciplinary approaches will be helpful for the quick, ultrasensitive analysis of these markers. Attempts should also be made to develop new CRP recognition molecules and new material to develop sensing platforms. While developing and implementing these concepts, care should be taken that these systems have promise for CRP analysis in body fluids.

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