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Research Article

Nonconjugated Polyelectrolyte as Efficient Fluorescence Quencher and Their Applications as Biosensors: Polymer-Polymer Interaction

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A simple fluorescence quenching method for the quantitation in serum of an acute phase reactant, C-reactive protein (CRP), which can differentiate between viral and bacterial infections, is described, where material and reagent costs are minimal. The study harnesses a fluorescence quenching between a nonfluorescent polyelectrolyte containing a ligand (O-phosphorylethanolamine, PEA) and fluorophore (fluorescamine Isomer 1) containing polyelectrolyte. The quenching was attributed due to strong polymer-polymer interaction through intermolecular hydrogen bonding. The nonlinear behaviour of Stern-Volmer plot indicates a binding induced quenching, that is, static quenching. However, fluorescence was found to increase in presence of C-reactive protein, due to the specific molecular recognition occurring between CRP and PEA, thereby excluding fluorophore containing chain. A definite correlation was found between concentration of CRP and fluorescence intensity and the method exhibited a linear relationship in the range of 40–360 ng/mL with a detection limit of 30 ± 2 ng/mL. The antibody free method was successfully applied for the analysis of CRP in human serum samples and the method showed good correlation with hospital measurements ($y = 1.0313x - 0.1423$; $n = 32$; $R = 0.9998$, $P < 0.0001$). Thus the fluorescence based polyelectrolyte biosensor is a potential system for rapid, and antibody free platform for CRP detection.

1. Introduction

Polyelectrolytes based fluorescent sensors have been an area of intense research for the past few decades, due to the interest in area such as controlled drug release, surface coating, and chemical and biological sensing [1–5]. Conjugated polyelectrolytes have received particular attention for sensing proteins, due to their water solubility and fluorescence properties [6–9]. Conjugated polyelectrolytes contain a set of structural attributes that make them useful in optical and electronic detection of many chemical and biological targets [10]. Fluorescence methods are being used for a variety of investigations in biochemical, medical, and chemical research. Fluorescence based sensors may rely on changes in emission intensity, life time, and wavelength (excitation and emission). However, fluorescence quenching, in which decrease of emission intensity and/or life time is measured, is one of the most common techniques in fluorescence based sensors because of its easy measurement and low detection limit.

Herein we report a nonconjugated polyelectrolyte as efficient fluorescent quencher and its application for the quantitation in serum of an acute phase reactant, C-reactive protein (CRP). In this investigation, we present a pioneering analytical platform integrating the properties of polyelectrolyte and fluorescence quenching mechanisms. As a platform model, measurement of CRP a cardiac inflammation marker was performed.

CRP is one of the well studied marker molecules in association with cardiovascular diseases (CD) [11]. CRP has been reported to be elevated up to 1000 folds during

many infectious states including myocardial infarction [12]. CRP consists of five identical subunits each of which is formed of 206 amino acid units [13]. It has binding sites for phosphocholine and its derivatives. The binding is known to be calcium-dependent. Recent studies are suggestive that CRP along with serum cholesterol is decisive factors in the initiation and progression of CD [14]. A growing body of evidence has suggested that considerable prospect exists in using CRP measurement as a predictor of possible cardiovascular risks.

Several methodologies have been reported for its estimation [15–18] and among the reported methods, immunoassays enjoy wide acceptance due to their specificity and sensitivity [19, 20]. One of the serious concern of these methodologies are associated with the often noted nonspecific binding and nonstability of the conjugated biomacromolecules (e.g., antibodies) [21]. Recently several measures have been taken to take care of these factors.

Efforts to develop techniques using nonbiological components, interestingly, are scanty. Merritt and Winkelman have reported an electrochemical sensor for CRP based on crown ether [22]. Apart from these report chemical sensors for the detection of CRP have not yet been reported. Here we report a new analytical platform for sensing CRP in human sera based on the fluorescence quenching of a pair of copolymers. The sensor platform described here could be modified for the detection of other clinically interesting molecules by the attachment of appropriate functionalities.

2. Experimental

2.1. Materials. Acrylic acid (Mw = 7206) and fluorescamine isomer 1 were purchased from Acros Organics, New Delhi, India. O-phosphorylethanolamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), human albumin, azobisisobutyronitrile (AIBN), and human C-reactive protein were obtained from Sigma-Aldrich chemicals Inc., Bangalore, India. These chemicals were used as received. Other analytical/chromatographic grade solvents were purchased from Merck or Sisco Chemicals, Mumbai, India

2.2. Homopolymerisation of Acrylic Acid. Briefly, acrylic acid (5 g) was homopolymerised in 50 mL benzene using AIBN as initiator at 60°C in an atmosphere of nitrogen. After 6 h, the homopolymer obtained (F0) was washed and dialysed against water and lyophilised.

2.3. Coupling of PEA to Polyacrylic Acid. 1 g of homopolymer was dissolved in water and the pH of the solution was adjusted to be around 4.5. Carbodiimide chemistry was used to form amide bond between carboxylic group of polyacrylic acid and primary amine group of PEA as depicted in Scheme 1(a) [23–25]. As a first step, EDC reacts with a carboxylic acid group and forms an amine reactive intermediate. In the next step, addition of a primary amine containing molecule in this reaction mixture leads to an amide linkage between amine and the activated carboxylic acid group. The activation is generally done at low pH (pH ~ 4.5) because at this pH acid group gets protonated. Biochemists use this procedure to link biomolecules or to modify biomolecule surfaces. PEA conjugated polymer was dialysed against water, lyophilized, and stored. The polymer was designated as F1.

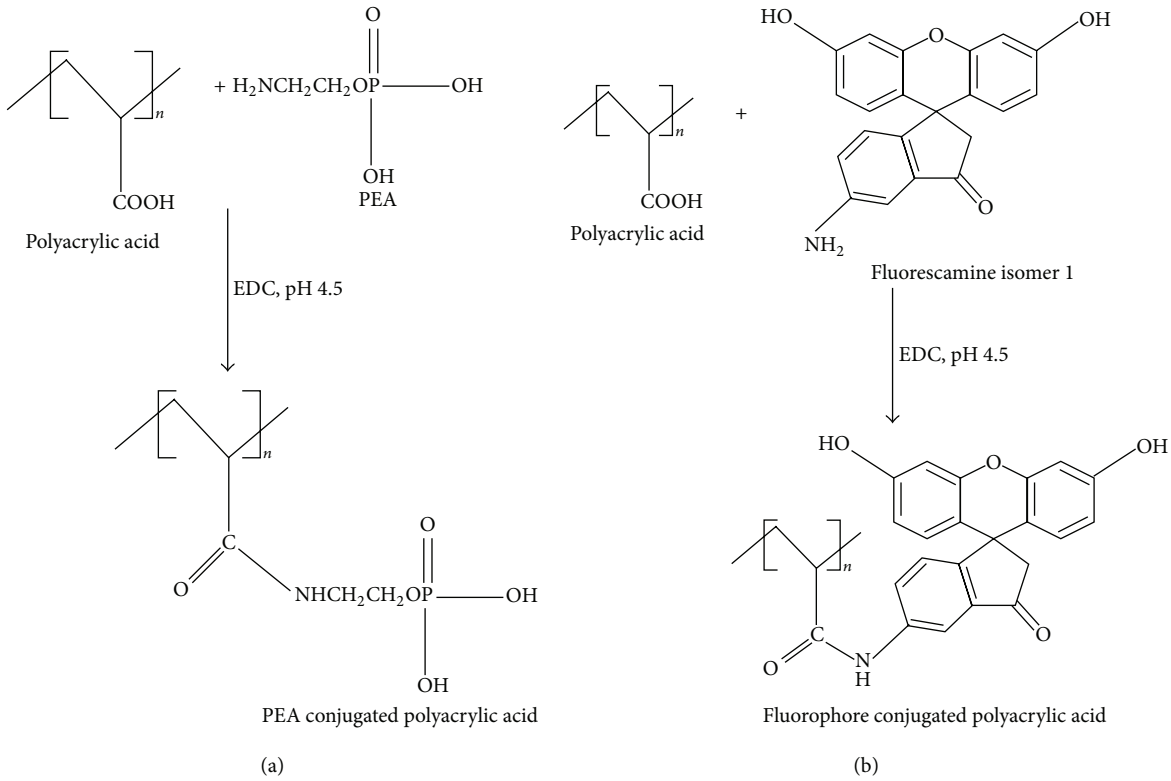
2.4. Coupling of Fluoreseamine Isomer 1 to Polyacrylic Acid. 1g of homopolymer was dissolved in water and the pH of the solution was adjusted to be around 4.5. Carbodiimide chemistry was used to form amide bond between carboxylic group of polyacrylic acid and primary amine group of fluorophore as depicted in Scheme 1(b). The fluorophore anchored polymer was dialysed against water and lyophilised. The polymer was abbreviated as F2.

2.5. Interaction of CRP with Polyelectrolytes F1 and F2. The analysis of protein interaction with the polyelectrolyte was performed by adding 20 mg of F2 in 50 mL of 0.1 M calcium chloride solution for the measurement of fluorescence. Exactly 20 mg of F1 was added to the same solution for the subsequent measurement. Varied amount of CRP were added to the solution (10 mL) containing mixture of F1 and F2. All the measurements were made in triplicate and the average readings were taken. The excitation wavelength was 496 nm and the emission was 520 nm. The relative intensities were corrected for dilution.

2.6. Interference Studies with a Mixture of Albumin, Fibrinogen, and γ Globulin. A 10^{-2} M stock solution of each protein (albumin, fibrinogen and γ-globulin) was prepared in phosphate buffer solution of pH 7. It is well known that concentration of albumin in the blood is 50 mg/mL. The levels of other proteins are relatively lower. Since the volume of serum we used for the measurement was 10 μL, we added a mixture of proteins (500 μg/mL) in which the concentration of albumin was kept at 480 μg/mL to assess the interference in the measurement of CRP.

2.7. Analysis of CRP in Human Sera. The levels of CRP in human sera were determined by two different methods: the fluorescence quenching method and by the method of nephelometry employing CRP specific antibodies. For the analysis of CRP by fluorescence method, ten microlitres of the serum separated from the blood samples were added into 2 mL of the copolymer solution. The copolymer solution was prepared by dissolving 10 mg each of polymers (F1 and F2) in 25 mL of phosphate buffer, pH 7, containing 0.1 M calcium chloride. The solution was shaken well and the fluorescence was measured at 22°C. All the measurements were made in triplicate and the average value was taken. Then, correlation between two methods was analysed with the Linear Fit of the Origin program.

2.8. Instrumental. The infrared spectrum was measured in the range 400–4000 cm^{-1} using a Nicolet Inc. (Madison, USA) model impact 5700 FTIR spectrometer with a horizontal ATR accessory containing diamond crystal. The numbers of scans were 50.



Scheme 1: (a) Schematic representation of formation of PEA conjugated polyacrylic acid by EDC chemistry and (b) schematic representation of formation of fluorophore conjugated polyacrylic acid.

The fluorescence spectrum was recorded using a Cary Eclipse model EL 0507 spectrofluorimeter.

Energy dispersive X-ray analysis was performed using an EDX model 6051 SP (Oxford Instruments, UK) attached to the scanning electron microscope.

MININEPH (Binding site Ltd., Birmingham, UK) was used for determining CRP concentration in serum using the principle of nephelometry. The light source is a diode laser that emits at 670 nm. The focussed light passes through a cuvette containing the reaction mixture, where antibody/antigen complexes cause light to be scattered. This scatter is proportional to the amount of antibody/antigen complexes that have formed and is detected by a photodiode. For each assay, a scatter is taken at the beginning of the antibody/antigen reaction (Blank), followed by a second scatter reading at a fixed time. The analyte concentration is calculated using the difference between these two readings. The measuring range is 3.5–112 mg/L (3.5–112 μg/mL).

3. Results and Discussion

Polyelectrolyte based sensory materials are one among them which has been widely used in the area of protein sensing or more precisely for biomolecular recognition. For the most part, this growth has been driven by the use of conjugated polyelectrolytes that are functionalised with appropriate side chains or ligands. Contrary to these reports we demonstrate the use of nonconjugated polyelectrolyte as fluorescence quencher and its application in the sensing of CRP. Towards this goal we have synthesised two polymeric formulations: (1) polyacrylic acid conjugated with PEA and (2) polyacrylic acid on conjugated with fluoreseamine Isomer 1.

3.1. Characterisation of Polyelectrolytes F1 and F2 (Fourier Transform Infrared Spectroscopy (FTIR)). Figure S1 (see supplementary information in the Supplementary Material available online at http://dx.doi.org/10.1155/2014/841857) shows the infrared spectrum of these polymers. PEA conjugated polymer (spectrum B) showed peak around 1553 cm^{-1} characteristic of amide bond (–CONH), confirming the coupling PEA onto polyacrylic acid through the activated carboxylic acid groups. The intensity of –CO and –OH groups were reduced substantially further reflecting the formation PEA conjugated entities through the carboxylic acid groups. The spectrum also showed a peak around 1000 cm^{-1} characteristic of PO_4^- moiety further confirming the result. Similarly the spectrum of fluorescamine conjugated polymer (spectrum C) showed distinctive peak around 1550 cm^{-1} characteristic of amide bond formation between the $-NH_2$ group of fluorophore and the carboxylic acid group of polyacrylic acid. A strong peak around 1110 cm^{-1}, characteristic of C–O–C of fluorescamine further indicates the formation fluorophore coupled polymer.

3.2. Energy Dispersive X-Ray Analysis (EDAX). EDX trace depicted in Figure S2 (Supplementary Information) shows the peak corresponding to phosphorous indicating the conjugation of phosphate moieties onto polyacrylic acid. Peaks of

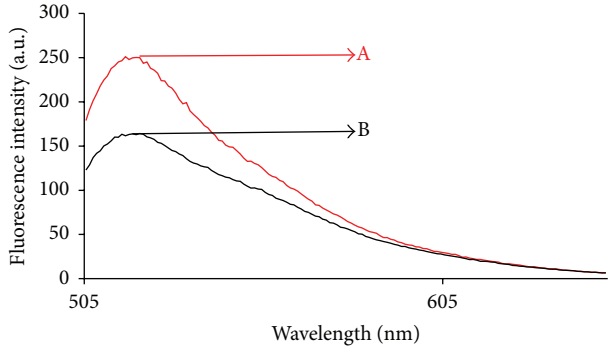


Figure 1: Fluorescence spectrum of F2 (trace A), fluorescence spectrum of F1 + F2 (trace B).

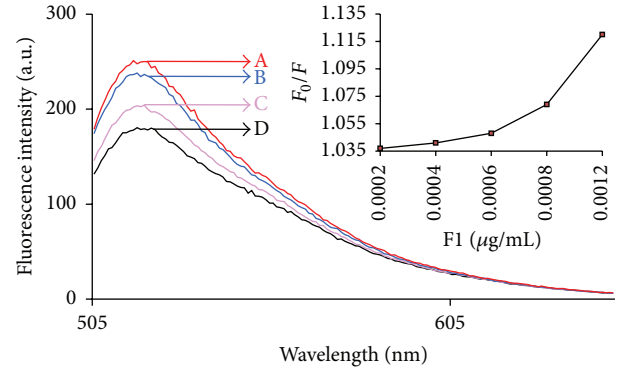


Figure 2: Fluorescence spectra in presence of different quencher concentrations. Inset is the Stern-Volmer plot.

Au and Pd can also be seen in the trace which is arisen from the coated gold layer prior to the observation.

3.3. Fluorescence Quenching Experiments. The emission spectrum of F2 is shown in Figure 1 (trace A). An equal quantity of F1 was added to the solution containing F2 and the reaction mixture was shaken well. The fluorescence measurements indicated a decrease in the fluorescence spectrum of F2 or in other words the fluorescence seems to be quenched (Figure 1, trace B). F1 quenched nearly 40% of the initial polymer fluorescence. The drastic quenching of polymer fluorescence of F2 by F1 could be traced due to strong polymer-polymer interaction. Kabanov et al. have studied in detail the association of polymeric chains in aqueous solution using fluorescence [26]. In our system of nonconjugated polyelectrolyte the possibility of energy transfer or electron transfer is completely ruled out. The quenching of fluorescence was attributed due to the strong interactions between polymeric chains due to intermolecular hydrogen bonding. Difference in quenching efficiency between different groups can be attributed to sterric, electrostatic, and hydrophobic interactions inherent to the differences in the molecular structure [27]. Here since PEA is a small molecule it is easily associated with the polymer structure.

Because of the charged groups on the backbone, chain-chain interactions in water soluble polyelectrolyte are subjected to some interpolymer forces due to hydrogen bonding. The interaction of these chains seems to curtail the fluorescence since the fluorophore is buried to an altogether different environment. When polyelectrolytes are mixed in water, the backbone of the two polymers interacts, which brings the fluorophore and PEA in close proximity to the optically active backbone. Under these circumstances, the fluorescence quenching of F2 by F1 is very efficient, or in other words when the two polyelectrolytes (F1 and F2) are mixed in water the system behaves as a "turn-off" sensor (Scheme 2(a)).

The fluorescence quenching efficiencies can be quantified using Stern-Volmer equation which is given by

$$\frac{F_0}{F} = 1 + K_{sv}[Q], \quad (1)$$

where F_0 and F are fluorescence intensities in the absence and presence of quencher and $[Q]$ is the quencher concentration.

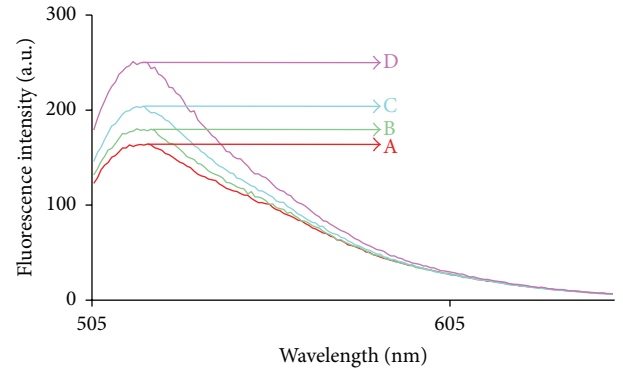
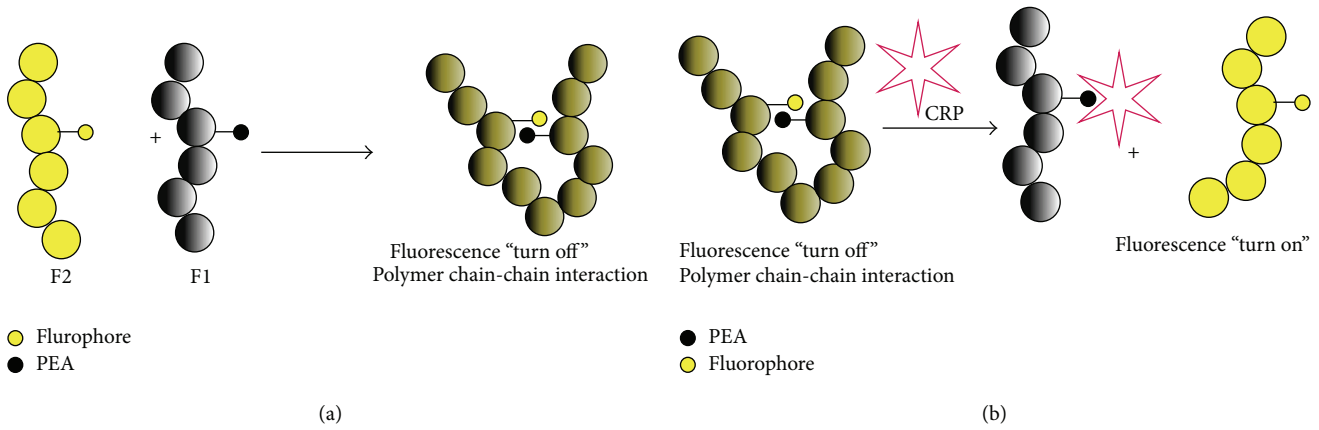


Figure 3: Effect of varied concentration of CRP on the fluorescence spectrum of F1 + F2. Fluorescence spectrum of F1 + F2 (trace A), fluorescence spectrum of F1 + F2 + 120 ng/mL CRP (trace B), fluorescence spectrum of F1 + F2 + 240 ng/mL CRP (trace C), and fluorescence spectrum of F1 + F2 + 360 ng/mL CRP.

The Stern-Volmer constant K_{sv} provides a direct measure of the quenching efficiencies and is determined from the linear portion of the plot F_0/F versus $[Q]$. Concentration-dependent quenching was used to generate a Stern-Volmer plot (Inset, Figure 2). For PEA induced fluorescence quenching, this value was found to be 1.8×10^2. The nonlinear behaviour of the plot indicates a binding induced fluorescence quenching that is, a static quenching.

3.4. Fluorescence Recovery by CRP. When F1 and F2 are allowed to interact with solution, the polyelectrolytes associate to give a fluorescence quenching effect as explained earlier. The addition of CRP, which selectively binds the PEA moieties on F1, was described to form a complex with PEA and thereby excludes the polymeric chain containing fluorophore. The specific molecular recognition between PEA and CRP draws away polyelectrolytic chain containing fluorophore, thereby recovering the emission (Figure 3).

In other words, the average distance between ligand and the fluorophore is larger upon addition of CRP since upon conjugation of PEA with CRP pulls the polymeric chain containing the ligand towards itself, and hence the polymeric chain containing the fluorophore is excluded thus



Scheme 2: (a) Schematic representation of "turn-off" sensor and (b) schematic representation of "turn-on" sensor system developed for CRP detection.

functioning as a "turn-on" system. A feasible mechanism for variation in fluorescence intensity is represented in Scheme 2(b). Regaining fluorescence emission upon addition of avidin has been reported by Shi and Wudl, in which the fluorescence emission of poly [lithium2-methoxy-5-(3′-propyloxylsulfonate)-1,4-phenylenevinylene] (Li^+-MPS-PPV) is quenched by a probe molecule [N-(biotinoyl)-N′-(acetyl 4,4′pyridylpyridinium iodide)] (BPP^+). The addition of avidin which selectively binds the biotin substructure encapsulate BBP^+ and draw it away from Li^+-MPS-PPV, thereby recovering the emission [28]. With different concentrations of CRP, the polymer fluorescence continues to grow up to [CRP] = 360 ng/mL. Further CRP addition (upto 360 ng/mL) does not result in additional changes in emission intensity. When all the binding sites on PEA are saturated with CRP, further addition of CRP results in the unperturbed fluorescence emission.

3.5. Effect of Nonspecific Proteins on the Fluorescence Quenching of F2 by F1. In a realistic analysis, a given sample may contain nontargets species that should not perturb the diagnostic signals of the sensory mechanism. Since PEA showed specificity for CRP binding, it was of interest to examine that other blood proteins would perturb the fluorescence. For the purpose of the study we chose three other blood proteins namely, albumin, fibrinogen, and γ-globulin. The addition of three different proteins to a quenched polymer solution (Figure 4, trace A) displayed unperturbed fluorescence emission as indicated in Figure 4 (trace B). Though we have not attempted to probe why albumin is repelled from the polymer surface, it seems that the presence of PEA moieties is responsible for the nonuptake of albumin. Park et al. have recently shown that binding of albumin onto polymeric nanoparticles could be reduced remarkably by modifying the surface by phosphorylcholine moieties [29]. Whereas addition of CRP to the same solution resulted in specific recovery of the emission as indicated in trace C (Figure 4). These results suggest that none of the blood proteins other than CRP has the capacity to bind to PEA, and thus the recovery seen from the polyelectrolyte/CRP solutions can only be due to the specific interaction between PEA and CRP moieties.

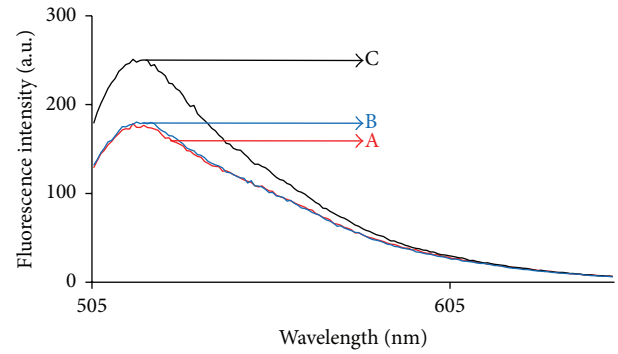


Figure 4: Fluorescence Spectrum of F1 + F2 (trace A), F1 + F2 + nonspecific proteins (trace B), and F1 + F2 + nonspecific proteins + CRP (trace C and D).

3.6. Measurement of CRP in Real Blood Samples. With the initial concept and development of new sensor platform, the next step was demonstrating the system in real blood samples. In order to accomplish this blood samples collected from various patients reported to the cardiology were tested using the method of polyelectrolyte and the values were compared with the values obtained for the same sample by normal CRP diagnostic test using CRP specific antibody. Fluorescence intensity increases only marginally when a negative CRP sample (samples containing CRP less than 3 mg/L) was added to the quenched polymer solution (Figure 5, trace B). However, in presence of positive samples (samples containing CRP higher than 3 mg/L), fluorescence intensity increases appreciably as depicted in Figure 5, traces C and D. The amount of CRP in various serum samples is summarised in Table 1. The quantity of CRP in these samples estimated using immunoassay is also shown in Table 1. The data suggest that the present method based on polyelectrolyte is indeed comparable with the well-known immunoassay technique.

3.7. Analysis of CRP in Human Sera: Correlation between Methods. The correlation between our method and conventional CRP nephelometry technique employed at our hospital

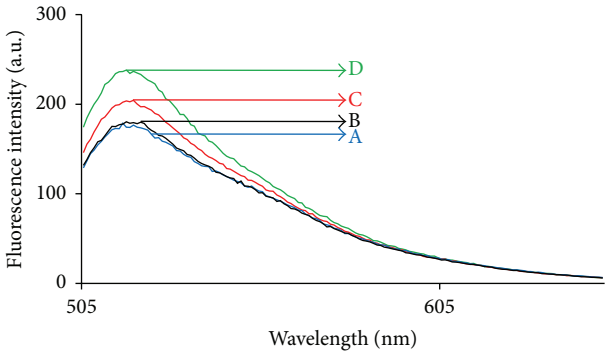


Figure 5: Fluorescence spectrum of F1 + F2 (trace A), F1 + F2 + negative serum samples (trace B), and F1 + F2 + positive serum samples (trace C and trace D).

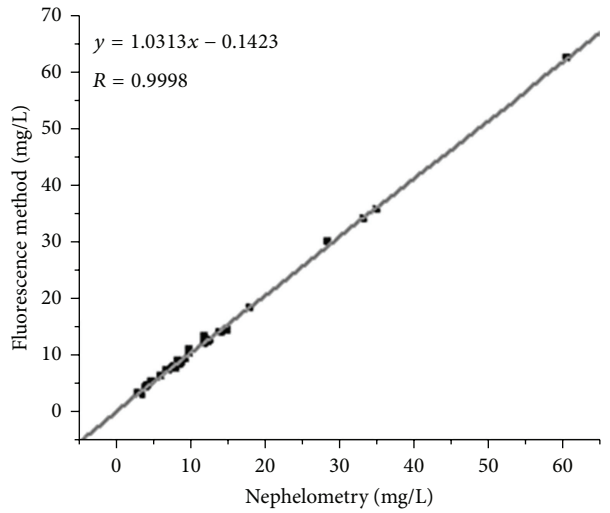


Figure 6: Analysis of CRP in human sera. Correlation between the CRP levels determined by nephelometry and by fluorescence was analysed by the Fit Linear of the Origin program ($n = 32$; $R = 0.9998$; $P < 0.0001$).

laboratory (Department of Biochemistry, Sree Chitra Tirunal Institute for Medical Sciences and Technology) was investigated. Accordingly, 40 blood patient samples (32 positive and 8 negative CRP samples) were determined by conventional nephelometry and by the method of fluorescence as described in Section 2. Then, correlation between the CRP levels determined by the method of nephelometry and those determined by fluorescence was analysed by the Fit Linear of the Origin program. As shown in Figure 6, the CRP levels of human sera determined by nephelometry showed a good correlation with those determined by fluorescence ($n = 32$; $R = 0.9998$; $P < 0.0001$) indicating the clinical relevance of our results. The correlation is described by the following equation: $y = 1.0313x - 0.1423$; $n = 32$. The mean difference of the methods according to the Bland and Altman plots is 0.4699 ± 6.45 ng/mL (Figure 7(a)). To get further insight into the affinity of PEA to CRP, binding affinity has been calculated from a dose-response curve constructed from the adsorption data by Langmuir fitting (Figure 7(b)). The binding constant

Table 1: Quantitative data of CRP in serum—comparison of the methods.

Sample code	Amount of CRP nephelometry (mg/L)	Amount of CRP fluorescence (mg/L)
1	4.58	4.73 ± 0.15
2	15.04	14.44 ± 0.09
3	14.11	14.25 ± 0.50
4	35.01	35.63 ± 0.10
5	28.66	30.17 ± 0.21
6	15.07	15.42 ± 0.23
7	12.84	12.76 ± 0.45
8	7.15	7.34 ± 0.12
9	12.00	13.45 ± 0.14
10	8.23	7.79 ± 0.05
11	8.76	8.76 ± 0.55
12	8.44	8.56 ± 0.09
13	33.45	34.10 ± 0.11
14	18.26	18.47 ± 0.41
15	7.06	7.45 ± 0.21
16	9.58	9.19 ± 0.10
17	14.12	14.21 ± 0.90
18	8.56	9.87 ± 0.15
19	10.07	10.98 ± 0.22
20	12.04	12.14 ± 0.55
21	4.82	4.97 ± 0.12
22	8.97	8.79 ± 0.15
23	17.72	17.64 ± 0.24
24	7.94	7.64 ± 0.11
25	4.95	4.25 ± 0.15
26	10.15	10.45 ± 0.20
27	16.29	16.45 ± 0.11
28	14.36	14.12 ± 0.16
29	12.48	12.24 ± 0.15
30	60.41	62.45 ± 0.24
31	12.97	12.21 ± 0.14
32	17.9	16.73 ± 0.09
33	Negative sample	Marginal change in fluorescence
34	Negative sample	Marginal change in fluorescence
35	Negative sample	Marginal change in fluorescence
36	Negative sample	Marginal change in fluorescence
37	Negative sample	Marginal change in fluorescence
38	Negative sample	Marginal change in fluorescence
39	Negative sample	Marginal change in fluorescence
40	Negative sample	Marginal change in fluorescence

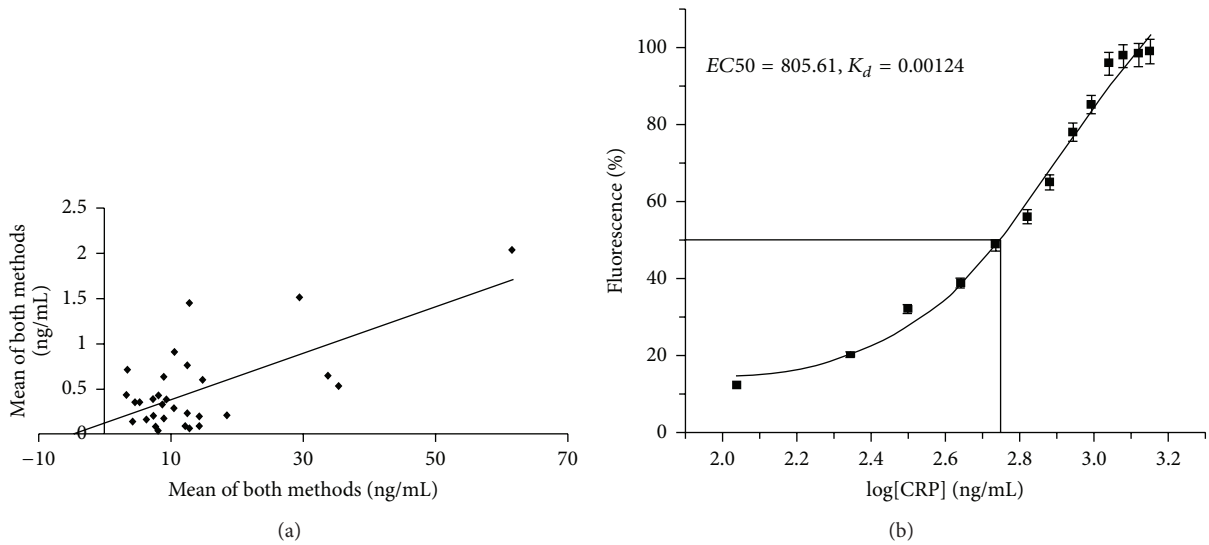


Figure 7: (a) Bland and Altman plots for measurement performed with nephelometry and fluorescence on human blood sera and (b) dose-response curve.

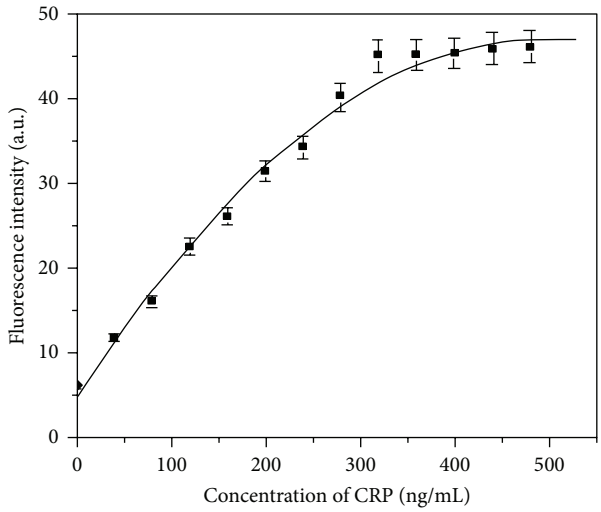


Figure 8: Graphical representation of variation of fluorescence intensity with concentration of CRP.

K_d was found to be 1.24×10^{-3} ng/mL. The low value of affinity constant indicates good binding affinity of PEA towards CRP.

3.8. Quantification of CRP. The fluorescence measurements of polyelectrolyte at different CRP concentrations were monitored. It was seen that, with increase in CRP concentration, the fluorescence intensity increases up to a certain level and ultimately it levels off indicating the saturation of the available binding sites for CRP (Figure 8). A linear relationship between concentration of CRP and fluorescence intensity was observed until the entire CRP was bound to PEA. Further addition of protein did not show any variation in fluorescence. A linear relationship was established in the range of 40–360 ng/mL. Further addition of CRP did not cause any change in fluorescence. So the present methodology could be useful for the detection of CRP in the range of 40–360 ng/mL with a detection limit of 30 ± 2 ng/mL. The lowest amount of CRP that can produce a variation in the fluorescence intensity was 30 ± 2 ng/mL which was taken as the detection limit of the method.

4. Conclusion

A new bioanalytical platform integrating the properties of polyelectrolyte and fluorescence quenching mechanism for the detection of C-reactive protein has been presented. Polyelectrolyte containing specific ligand for CRP and fluorescamine were synthesised. The fluorescence emission of fluorophore conjugated polyelectrolyte was found to decrease in presence of polyelectrolyte containing CRP specific ligand. The substantial reduction in the fluorescence intensity was attributed due to the strong polymer-polymer interactions. The fluorescence intensity was, however, found to increase in presence of CRP indicating the possibility of using this system for the detection and estimation of CRP. Other blood proteins were found not to have any interference in the measurement. The method exhibited a linear relationship in the range of 40–360 ng/mL with a detection limit of 30 ± 2 ng/mL. Fluorescence measurements were used to estimate CRP in serum and the results showed good correlation with hospital measurements by nephelometry. Although this work demonstrated the detection of a clinically interesting plasma protein in serum, the sensor platform described here could be modified for the detection of other clinically interesting substances. In addition to the model studied, the ease of performance, very less instrumentation, and low cost suggest broad applicability of this method. Future studies could be focussed to integrate the findings to the level of a technology, so that it could be adopted into any clinical laboratory where resources are minimal to build high tech infrastructure.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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