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Chemiluminescence detection of label-free C-reactive protein based on catalytic activity of gold nanoparticles

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ABSTRACT

A novel, quantitative analytical method for measuring C-reactive protein (CRP) levels in human serum has been developed based on the catalytic activity of gold nanoparticles (GNPs) and luminol–H_2O_2 chemiluminescence (CL). The CL intensity in the presence of CRP and its ligand, *O*-phosphorylethanolamine (PEA), was greatly enhanced due to the aggregation of GNPs after the addition of 0.5 M NaCl. Any pretreatment steps, such as covalent functionalization of GNPs, addition of antibodies, or labeling of CRP, were not needed for CL detection. The CL enhancement was linearly proportional to CRP concentration in the range of 1.88 fM to 1.925 pM. The detection limit of CRP in serum samples was estimated to be as low as 1.88 fM. The detection sensitivity was increased more than 164 times of magnitude over that of the conventional, enzyme-linked immunosorbent assay (ELISA) method. This proposed GNP-based CL detection method offers the advantages of simplicity, rapidity, and sensitivity.

1. Introduction

Chemiluminescence (CL) is a production of electromagnetic radiation by a chemical reaction, which is proportional to the concentration of a limiting reactant involved in the reaction [1,2]. CL has been exploited in a wide range of applications due to its high sensitivity, wide calibration range, and suitability for miniaturization. Recently, CL-based detection has attracted attention for the analysis of protein and DNA [3–5].

Gold nanoparticles (GNPs) have been widely used in recent decades; their catalytic activity in gas phase and liquid phase redox reactions is a new area of research [6]. It has been reported that there might be several effects contributing to the special catalytic properties of supported nano sized gold particles. The most important effect is related to the availability of many poorly coordinated gold atoms on the small particles. Interaction between oxide materials and GNPs, therefore, is not essential for the catalytic activity of GNPs [7]. Cui et al. have also found that GNPs of different sizes could act as catalysts to enhance the luminol–H_2O_2 and bis(2,4,6-trichlorophenyl) oxalate-hydrogen peroxide CL reactions [8]. Some unsupported GNPs such as gold colloids, might be directly used as catalysts for some CL reactions in liquid phase systems [9,10]. Niazov and coworkers have introduced DNAzyme functionalized Au-NPs as biocatalytic conjugates for the generation of CL in the presence of hemin, H_2O_2 and luminol. The system has been applied to sense DNA or telomerase activity [11]. In addition, Qi et al. have reported that catalytic activity of GNPs greatly enhanced after it was aggregated by 0.5 M NaCl in luminol–H_2O_2 system. They proposed a method for CL detection of label free and homogenous DNA hybridization [12].

On the other hand, optical sensing based on the plasmon resonance absorption exhibited by nano-particles has also been used in the context of developing analytical tools for clinical diagnosis [13,14]. In particular, extensive studies have been reported on the use of GNPs as sensing platforms that exploit the plasmon resonance detection method for bio-specific interaction analysis as well as biomolecular interaction assays [15,16]. A small change in the size, shape, local environment, surface nature, and degree of aggregation of nanoparticles leads to tunable changes in their optoelectronic properties that, in fact, enabled their use in sensing and quantification of molecules of interest [17]. In addition, biomarker measurement in the blood serum is indispensable in fundamental research and clinical diagnosis. It is well recognized that many diseases are caused by variations in functional proteins. Therefore, there is a great need for measurement of these proteins for the successful treatment of life-threatening ailments such as cardiac diseases. For example, C-reactive protein (CRP) is a common indicator during inflammation processes [18]. Since elevated CRP can be linked to the incidence or strictness of heart attacks, strokes, and other cardiovascular diseases, CRP has been employed to assess the risk of cardiovascular diseases [19].

Several methodologies have been reported for the estimation of CRP [20–22]. Among the currently used techniques, immunoassays enjoy wide acceptance due to their specificity and sensitivity

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[23]. These techniques, using specific antigen–antibody interactions, have been regarded as extremely useful approaches for the routine assay of CRP. Most of them are fluorescence-based or biochip-based and involve complicated labeling steps, immobilization on solid support, and are time consuming and costly [24–27]. In contrast, efforts to develop techniques using non-biological components, for instance a GNP-enhanced chemiluminescence-based CRP detection approach, have not been well reported until now. Recently, Raj et al. reported a colorimetric sensing method for the detection of CRP in serum using 16-mercaptohexadecanoic acid-functionalized gold nano-particles. They observed color change and a decrease in absorption in the plasmon peak of the modified GNP dependent upon CRP concentration [28].

In this study, we demonstrate a GNP-based luminol–H_2O_2 liquid-phase CL system for estimation of CRP in serum samples. Although gold colloids have been used in some liquid-phase CL systems, real applications of this novel catalytic CL phenomenon are finite [29]. It is necessary to explore some new applications of gold colloids in liquid-phase CL systems, which may open new possibility for detection and quantification of biomarkers in clinical samples without labeling or the use of antigen-antibody interaction. Moreover, this method should be a reliable and sensitive alternative to conventional enzyme-linked immunosorbent assay (ELISA). To achieve this goal we have employed an entirely different CL designing strategy. A surprising phenomenon was observed; the catalytic activity of both the anionic and cationic GNPs in a luminol–H_2O_2 CL system were greatly enhanced when GNPs were aggregated by NaCl after addition of CRP with its ligand (PEA) in the same solution. By taking advantage of this phenomenon, a label-free CL detection technique for CRP quantification in human serum samples is proposed.

2. Materials and methods

2.1. Chemicals and reagents

Chloroauric acid ($HAuCl_4$), sodium citrate, sodium borohydride, 2-aminoethanithiol, luminol (3-aminophthalhydrazide), sodium hydroxide, sodium chloride, calcium chloride, *O*-phosphorylethanolamine (PEA), cytochrome *c*, and albumin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Recombinant human CRP (stock solution, 100 μg/mL, in 1× phosphate buffered saline (PBS) and 0.1% bovine serum albumin) and an ELISA kit were received from R&D Systems (Minneapolis, MN, USA). Buffer solutions were prepared with ultra-pure water (>18 MΩ), filtered through a 0.2 μm membrane filter (Whatman International Ltd., Maidstone, England), and photo-bleached overnight by a UV lamp (G15TE, Philips, The Netherlands). Standard CRP solutions of various concentrations were diluted in 1× PBS/0.01 M calcium chloride solution (pH ~7.4) prior to use. A 10 mM stock solution of luminol was prepared by dissolving 443 mg luminol in 250 mL of 0.1 M NaOH solution without purification. A working solution of 0.5 mM luminol was prepared by diluting the stock solution, and a working solution of 1 mM H_2O_2 was prepared fresh daily from 30% (w/w) H_2O_2.

2.2. Synthesis and characterization of GNPs

Anionic GNPs were synthesized followed by citrate reduction according to a previously published method with slight modifications [30]. Briefly, 100 mL of 1 mM $HAuCl_4$ solution was heated just to its boiling point. Afterward, 5 mL of 5 mM trisodium citrate solution was added quickly with vigorous stirring, and then boiled for 10 min. The color of the solution changed from yellow to wine-red. Then, it was cooled to room temperature and stored at 4 °C before use. In this method, sodium citrate serves both as a reducing agent and an anionic stabilizer.

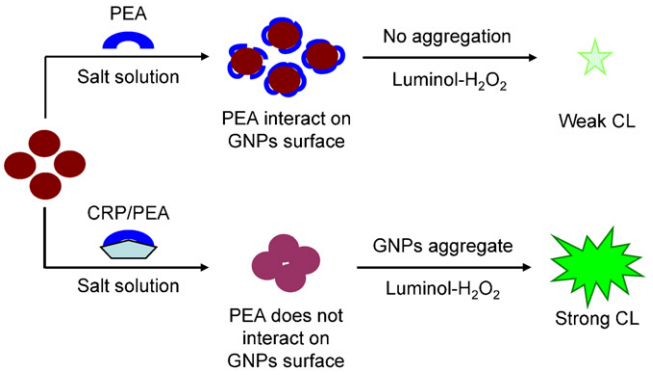

Fig. 1. A schematic diagram for GNP-based CL quantification of label-free C-reactive protein in serum. *Acronyms*: CL, chemiluminescence; CRP, C-reactive protein; GNPs, gold nanoparticles; and PEA, *O*-phosphorylethanolamine.

Cationic GNPs were prepared by reducing $HAuCl_4$ with $NaBH_4$ in the presence of 2-aminoethanethiol [31]. To 50 mL of 1 mM $HAuCl_4$ solution 1 mL of 200 mM 2-aminoethanethiol was added then stirred with a magnetic bar for 20 min at room temperature. Subsequently, 25 μL of 10 mM $NaBH_4$ solution was added, stirred vigorously for 15 min in the dark, then stored at 4 °C. A UV–vis spectroscope (UV-3600, Shimadzu, Columbia, USA) and transmission electron microscope (TEM, JEM2200-FS, Jeol, Japan) were used to explore surface plasma resonance bands and sizes of the synthesized GNPs, respectively.

2.3. Luminol–H_2O_2 chemiluminescence detection

A schematic diagram for GNP-based CL quantification of label-free C-reactive protein in serum is shown in Fig. 1. A standard CL detection was performed following two steps. First, 100 μL of 0.1 mM PEA (ligand of CRP) solution was mixed with 100 μL of GNPs in a 96-well Costar® flat-bottom plate. Then 100 μL of 0.5 M NaCl salt solution was added and mixed well followed by the injection of 200 μL of luminol–H_2O_2 solution (0.5 mM luminol and 1 mM H_2O_2 = 2:1, v/v); the CL signal was measured and recorded.

Second, a 0.1 mM PEA solution was added in CRP standard solution (1:2, v/v). The CRP standard solution was diluted to various concentrations ranging from 1.925 pM to 1.88 fM with 1× PBS/0.01 M calcium chloride solution. At this concentration of Ca^{2+}, the CRP showed high sensitivity to bind with its ligand (PEA) [32]. Then, 100 μL of CRP/PEA complex was added to 100 μL of GNPs. The serial dilution technique was followed with an initial CRP concentration of 1.925 pM. Subsequently, the salt solution and luminol–H_2O_2 solution were mixed as noted above and CL intensity was measured. Standard CL intensity was measured using a luminometer (TriStar multimode microplate reader, LB 941, BERTHOLD TECHNOLOGIES GmbH & Co. KG, Germany). As a sample reservoir, 96-well Costar® flat-bottom plates were used.

Spectral intensity changes without CRP and after addition of CRP with PEA were measured by a spetrofluorometer (FP-6500, Jasco Corporation, Tokyo, Japan) equipped with a Xn lamp 150 W light source, operating from 220 to 750 nm both for emission and excitation, with a quartz cell sample holder. All sample solutions were mixed sequentially as stated above, and the spectral changes in luminescence intensities were recorded without CRP and after CRP treatment. Moreover, absorption changes of the GNPs before and after treatment with PEA or CRP/PEA solution were monitored using a UV–vis spectroscope. In order to evaluate the potential for consistent use of this assay in clinical applications, human serum samples with unknown CRP levels were used instead of the

standard CRP following the same procedures. Other proteins like albumin and cytochrome *c* were used as controls to confirm the accuracy of the obtained results.

2.4. Preparation of human serum samples

Patients suffering from heart disease, inflammation, and tissue injury, as well as healthy individuals of different ages gave their consent for samples of their blood to be taken. Blood was drawn from individuals into non-clotting, agent-free vacutainers, and the serum was separated using standard clinical procedures. Briefly, the samples were centrifuged at 1500 rpm for 10 min at 2 °C. The isolated serum samples were stored at −20 °C prior to use.

3. Results and discussion

3.1. Characterization of synthesized GNPs

UV–vis spectroscopy was used to explore the change in absorption of the GNPs induced by CRP and its ligand, PEA, in aqueous solution. The GNPs in aqueous solution were stabilized against aggregation by the negative capping agent's (citrate ions or hydroborate ions) electrostatic repulsion against van der Waals attraction between GNPs [33], and exhibited a surface plasma resonance band located at about 531 nm for the anionic GNPs (curve "i" in Fig. 2A) and 528 nm for the cationic GNPs (curve "i" in Fig. 2B). Li and Rothberg reported that the addition of enough salt (i.e., 0.5 M NaCl) screens the repulsion between the GNPs and easily causes the aggregation of GNPs. As a result, the aggregated GNPs showed a broad surface plasma resonance absorption over the range of 550–850 nm [34]. GNPs only in the presence of PEA (without CRP) caused a slight red shift of 7 and 8 nm for the absorption peaks at 538 nm and 536 nm for anionic and cationic GNPs, respectively (curve "iii"). This indicates the interactions of PEA on both GNP surfaces. After addition of the CRP/PEA complex, the GNPs had a broad, featureless absorption spectrum (curve "ii" in Fig. 2A and B), which suggested the aggregation of GNPs.

These results were consistent with TEM images (Fig. 3) that were obtained to confirm the dispersal and aggregation of GNPs before and after the addition of CRP with its ligand (PEA). Moreover, the average diameters of both the freshly synthesized, anionic and cationic GNPs were measured as 31 ± 3 and 27 ± 2 nm, respectively. GNPs lingered in a dispersed state in aqueous solution as nanoparticles (image "i" in Fig. 3A and B). Both anionic and cationic GNPs in the presence of only PEA were highly dispersed (image "ii" in Fig. 3A and B). Although a sufficient concentration of NaCl (0.5 M) was added, there was no aggregation because PEA contains both a nitrogen atom-containing amino group and a negatively charged phosphate group in its backbone structure. It has been reported that there is a stronger coordination interaction between the nitrogen atom of an amino group and a Au (gold) atom in aqueous solution than the electrostatic repulsion between the negatively charged phosphate group and negatively charged capping agents (like citrate ions) of GNPs [35]. Therefore, the negative charge on the backbone was sufficiently distant so that attractive van der Waals forces between the amino groups and gold nanoparticles were adequate to cause PEA to stick to the gold surface [36]. As a result, no aggregation was observed in the presence of NaCl salt solution. After mixing of CRP/PEA complex into the GNP solution, significant aggregations of both GNP types were found ("iii" in Fig. 3A and B). These results are caused by the fact that CRP molecules in 1× PBS/0.01 M calcium chloride solution (pH ~7.4) show high affinity for binding with the calcium-mediated binding site of its ligand PEA; thus the formation of the CRP/PEA complex occurs when CRP and PEA are combined in buffer solution before the addition of GNP solution [37]. Under this condition, the addition of salt (NaCl) would screen the repulsion between the GNPs and easily induce GNP aggregation. As a result, the aggregated GNPs showed broad spectra of surface plasma resonance absorption (Fig. 2).

3.2. Optimization of the CL reaction conditions

Some experimental parameters such as concentration of luminol, H_2O_2, pH, etc. were optimized for the CL reaction. The concentration of luminol is an important factor affecting signal intensity. To investigate the effect of luminol concentration on CL emission intensity a study was performed in the range of 0.1–2.5 mM luminol. The greatest CL emission intensity was obtained at 0.5 mM luminol under our applied conditions (Fig. S1). Regarding the effect of hydrogen peroxide (H_2O_2) concentration on relative CL intensity, the maximum CL emission was found at 1 mM H_2O_2 solution. Because of the nature of the luminol reaction, which is more favored under basic conditions, an alkaline medium (0.1 M NaOH, pH 12) was used to improve the sensitivity of the system. Varying GNP size in the range of 6–99 nm linearly increased the CL intensity. This result confirmed the size of GNPs also affects CL intensity [8,9]. In our experiments, the diameters of both anionic and cationic GNPs were 31 ± 3 and 27 ± 2 nm, respectively.

There was a very low CL signal with a low concentration of NaCl because of strong electrostatic repulsions between the phosphate group of PEA and the anionic groups of capping agents in the GNP solution. GNPs would not aggregate in low concentrations of NaCl. The CL intensity rose slowly at first between 0.1 and 0.4 M NaCl, then more rapidly, and remained almost constant above 0.5 M (Fig. S2). Finally, we selected 0.5 M NaCl, which was convenient for CL detection. Although we used another salt ($CaCl_2$) in the PBS buffer for diluting the CRP samples, it had no effect on GNP surface resonance or intensity, which was consistent with an earlier report [38].

3.3. Catalytic activity of GNPs on luminol–H_2O_2 CL signal tempted by CRP

In the absence of CRP, its ligand PEA could adsorb onto the GNPs' surface due to strong coordination interaction, thereby shielding the aggregation of GNPs at 0.5 M NaCl. The dispersed GNPs induced a weak CL signal from the luminol–H_2O_2 system (Fig. 1). Upon allowing CRP to react with its ligand PEA the CRP/PEA complex was formed. When the CRP/PEA complex was injected into the GNP solution at 0.5 M NaCl aggregation of GNPs resulted, and the aggregated GNPs showed a strong CL signal. Compared to the CRP estimation by conventional ELISA, our approach possessed some remarkable features: (1) it did not require labeling, (2) the protocol avoided the strict stripping procedure of metal nanoparticles, and (3) the method was very simple. The detection limit (S/N = 3) of CRP was estimated to be as low as 1.88 fM. The sensitivity was increased more than 164 times of magnitude over that of a colorimetric ELISA method.

The luminol–H_2O_2 CL system is a popular and well-reported CL system that emits CL at ~425 nm in alkaline solution [39,40]. In addition, GNPs have already been used as catalysts for some CL liquid-phase reactions [9,41,42]. It was found that, in the range of 6–99 nm, GNPs could enhance the luminol–H_2O_2 CL to different degrees [9]. Considering the catalytic properties of GNPs should be influenced by its protecting agents, it was necessary to investigate the CL behavior of the luminol–H_2O_2-GNPs system after addition of PEA or CRP/PEA complex. In the absence of GNPs, luminol–H_2O_2 gave a weak CL signal (curve "i" in Fig. 4A and B). When 100 μL of GNPs was added into this CL system, however, the CL signal was enhanced (curve "ii" in Fig. 4A and B). When 100 μL of 0.1 mM PEA was injected into the GNP-containing luminol–H_2O_2 system,

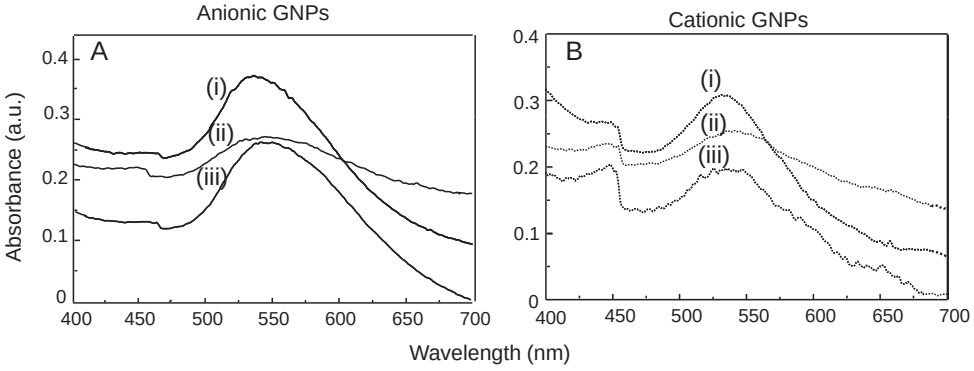

Fig. 2. UV–vis absorption spectra using anionic (A) and cationic (B) GNPs. Curve (i) in both figures represents the absorption of freshly prepared GNP solution without adding PEA or CRP/PEA. Curve (ii) represents GNPs/PEA/CRP with NaCl solution, and curve (iii) was generated using GNPs/PEA with NaCl solution. Experimental conditions: NaCl, 0.5 M; PEA, 0.1 mM; and CRP, 1.925 pM.

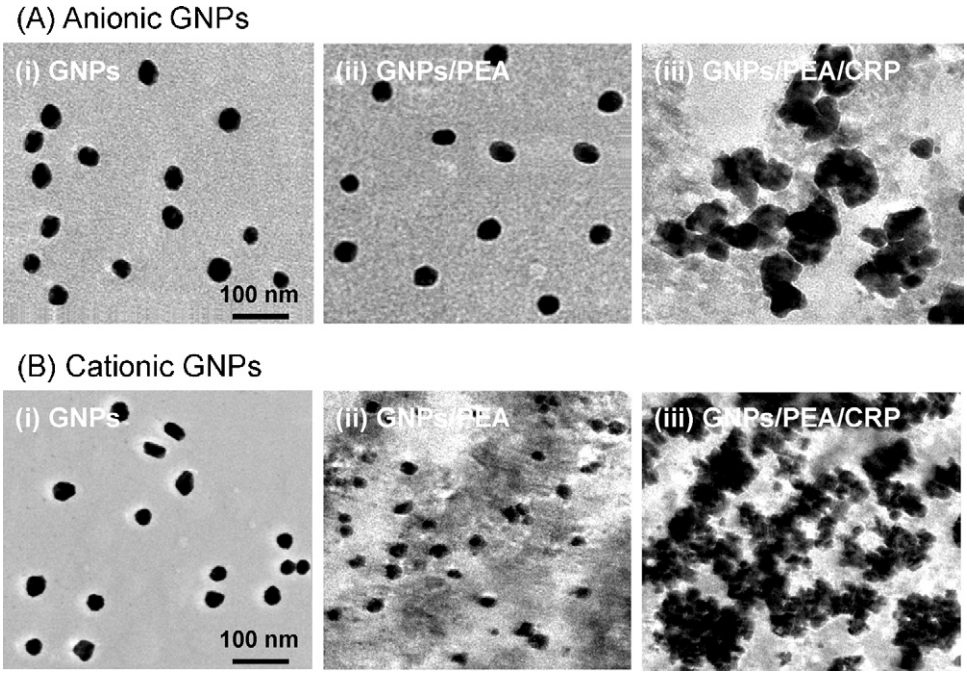

Fig. 3. Representative TEM images using anionic (A) and cationic (B) GNPs. In both figures, image (i) represents GNP solution; (ii) GNPs/PEA + NaCl; (iii) GNPs/PEA/CRP + NaCl. Experimental conditions: GNPs, 1 mM; PEA, 0.1 mM; CRP, 1.925 pM; and NaCl, 0.5 mM.

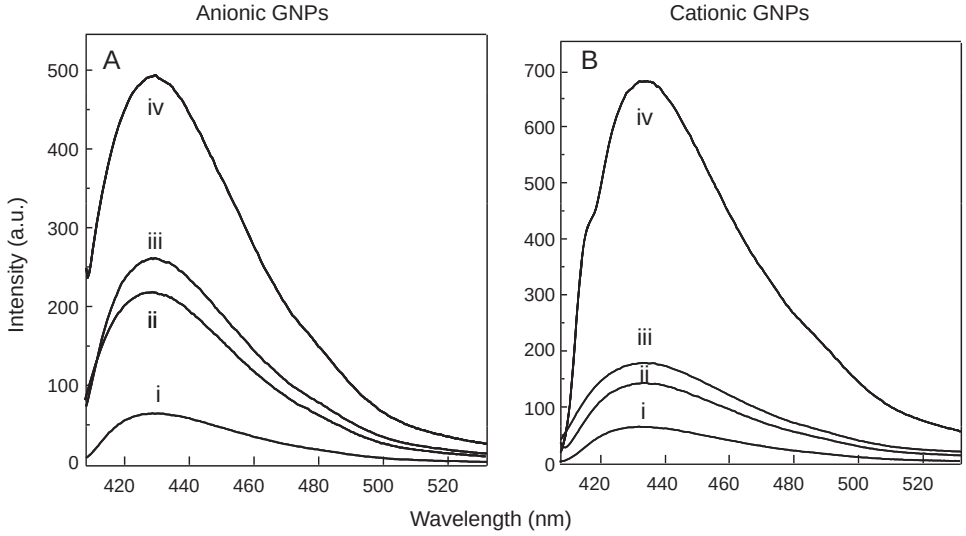

Fig. 4. Effect of (A) anionic and (B) cationic GNPs on the luminol–H_2O_2 CL spectral intensity (i) luminol–H_2O_2; (ii) luminol–H_2O_2/GNPs; (iii) luminol–H_2O_2/GNPs/PEA with NaCl; (iv) luminol–H_2O_2/GNPs and PEA/CRP complex with NaCl. Experimental conditions: Luminol, 0.5 mM; H_2O_2, 1 mM; PEA, 0.1 mM; NaCl, 0.5 M; and CRP, 1.925 pM.

however, a slightly increased CL signal was obtained (curve "iii" in Fig. 4A and B). The CL intensity increased dramatically (curve "iv" in Fig. 4A and B) when the CRP/PEA solution was added to the GNPs and luminol–H_2O_2 system. This CL enhancement was proportional with various concentrations of CRP (1.88 fM–1.925 pM) (Fig. S3A and B). The maximum signal intensities, 596 a.u. at 424 nm for anionic and 684 a.u. at 428 nm for cationic GNPs, were observed using 1.925 pM CRP with a fixed concentration of other components, as stated in Fig. 4. It can therefore be assumed that GNPs catalyze the luminol–H_2O_2 CL reaction and greatly enhanced its catalytic power in the presence of CRP in the same solution. Previously, we confirmed from TEM data (Fig. 3) that in the presence of only PEA, GNPs remain in a dispersed state, and when CRP/PEA are added together aggregation results. Furthermore, from CL spectral intensity, it was observed that the aggregated GNPs resulting from the addition of CRP/PEA can enhance luminol–H_2O_2 CL more than the dispersed GNPs. Since the interaction of GNPs with luminol or H_2O_2 can be affected by negative surface charge and binding area on the GNPs' surfaces, the enhancement effect of the aggregated GNPs may be due to a change in the GNPs' surface properties that alters that interaction. A comparison of the negative surface charge density of the dispersed GNPs with the aggregated GNPs revealed that the negative surface charge density of the dispersed GNPs is rather high [12].

In alkaline conditions (pH 12), hydroperoxide ion (HO_2^-) is easily formed from H_2O_2 in the luminol–H_2O_2 CL system [43]. Similarly, the luminol anion is the main molecular form of luminol in strong basic media. The anionic HO_2^- (oranionic luminol) would not easily interact with the anionic GNPs because of electrostatic repulsion. So, the dispersed GNPs with high negative surface charge density resulted in a rather low catalytic activity in the luminol–H_2O_2 CL reaction. After injection of CRP/PEA and NaCl solution, the salt solution screened the repulsion between the negatively charged GNPs to each other and led to GNP aggregation. The aggregation of the GNPs decreased the negative surface charge density as compared to the dispersed GNPs, thus favoring the interaction between anionic HO_2^- (or anionic luminol) and GNPs. So, the aggregated GNPs showed a higher catalytic activity in the luminol–H_2O_2 CL reaction than the dispersed GNPs (Fig. 4). On the other hand, the oxidation state of Au is not involved in the catalysis process of the CL reaction [12].

Furthermore, the cationic GNPs would be expected to show a higher catalytic activity than the anionic GNPs because the anionic HO_2^- (or anionic luminol) could easily interact with the cationic GNPs thus forming large clusters. Therefore, we obtained stronger CL emission (684 a.u.) using the cationic GNPs compared with the anionic GNPs (596 a.u.). Similar CL intensity variation was obtained by measuring CL at various steps of the working procedure as described in the next section. Therefore, we concluded that the decrease in the negative surface charge density and highly aggregated state of the GNPs would increase the catalytic activity of the luminol–H_2O_2 CL system.

3.4. Measurement of luminol–H_2O_2 CL intensity and quantification of human CRP

Under the optimized experimental conditions, the relationship between the CL intensity and the concentration of CRP strands was investigated using a luminometer and 96-well Costar® flat-bottom luminometer plates as a sample reservoir. Average CL intensity of anionic and cationic GNPs after adding 100 μL of 0.5 mM luminol–H_2O_2 complex was 56,129 and 66,037 a.u., respectively. The addition of only PEA in luminol–H_2O_2 with GNPs resulted in a nearly identical CL intensity. The CL intensity of luminol–H_2O_2 in the GNP solution increased with increasing CRP concentration from 1.88 fM to 1.925 pM after mixing

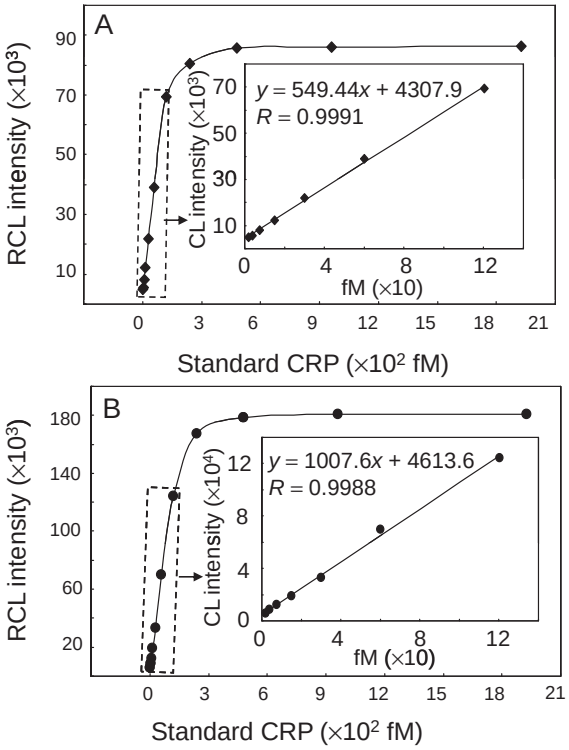

Fig. 5. Calibration curves using relative chemiluminescence (RCL) intensities of the luminol–H_2O_2 CL system for (A) anionic GNPs and (B) cationic GNPs (shown in Table 1) at various concentrations of CRP (1.88 fM–1.925 pM). Linear ranges both for anionic and cationic GNPs are 1.88–120.31 fM.

with PEA (Fig. 5). Relative CL intensity was calculated by subtracting the CL of GNPs with luminol–H_2O_2 from the CL of the CRP/PEA complex (Table 1). The fitted regression equations of the calibration graph were $y = 549.44x + 4307.9$ ($R = 0.9991$) and $y = 1007.6x + 4613.6$ ($R = 0.9988$) for both the anionic and cationic GNPs respectively, with a dynamic range of 1.88 fM–1.925 pM (Fig. 5). The maximum CL intensity was measured as 86,349 a.u. for anionic and 181,022 a.u. for cationic GNPs (Table 1). CRP concentrations below 1.88 fM yielded the same or less than the intensity value of the luminol–H_2O_2 system without CRP (56,129. and 66,037 a.u.

Table 1
CL intensities at various concentrations of standard CRP using anionic and cationic GNPs.

Concentration of CRP standard (fM)	Relative CL intensity of CRP/PEA in anionic GNPs	Relative CL intensity of CRP/PEA in cationic GNPs
1925	86,349	181,022
962.5	86,129	180,727
481.25	85,616	178,670
240.62	80,463	167,251
120.31	69,387	124,138
60.16	42,039	69,724
30.08	24,929	33,082
15.04	12,324	19,099
7.52	8,095	12,389
3.76	5,424	8,837
1.88	3,942	5,608
Cytochrome *c*	No significant change	No significant change
Albumin	No significant change	No significant change

The average CL intensities of anionic and cationic GNPs after adding 100 μL of 0.5 mM luminol–H_2O_2 complex were 56,129 and 66,037 a.u., respectively. Relative CL was measured by subtracting the CL of luminol–H_2O_2 in the GNP solution from the CL after the addition of the CRP/PEA complex in luminol–H_2O_2 containing both types of GNPs. Finally, these values were used to plot a calibration graph shown in Fig. 5. The serial dilution technique was used for CRP standard sample dilution.

Table 2
Estimated CRP concentrations in various human serum samples using the GNP-based luminol–H_2O_2 CL detection method and conventional ELISA.

Serum sample	1	2	3	4	5	6	7	8	9
Anionic GNPs (pM)	9.7	14.9	12.3	28.7	22.9	27.7	18.3	15.4	26.4
Cationic GNPs (pM)	9.6	14.1	11.9	28.4	21.9	27.4	18.2	15.9	26.3
ELISA (pM)	8.5	15.1	10.8	26.5	19.7	30.4	17.4	14.3	22.4

Comparison between the obtained results of both methods using Student's *t*-test; mean = 1.24, standard deviation = 1.94, $t_{calculated}$ = 2.0 and mean = 0.96, standard deviation = 1.97, $t_{calculated}$ = 1.5 ($<t_{table}$ = 2.262 at the 95% confidence level) both for anionic and cationic GNPs, respectively.

for anionic and cationic GNPs, respectively). Therefore, we assumed the LOD under the applied conditions was 1.88 fM, which was also verified by spectral CL intensity using a spectrofluorometer (Fig. S3A and B). The linear equation in Fig. 5 was used for calculating unknown CRP concentrations in human serum samples.

The specificity of the present luminol–H_2O_2 CL intensity-based assay was examined by detecting the CL response using two blood proteins, albumin and cytochrome *c*. To the solution of luminol–H_2O_2 and GNPs, a 100 μL volume albumin/PEA or cytochrome *c*/PEA was added and the CL intensity was monitored; the concentration of both proteins was 3.85 pM (two-fold higher than maximum CRP concentration, 1.925 pM). The intensity was unchanged and nearly equal to the luminol–H_2O_2/GNPs CL intensity without CRP (Table S1). This result indicates that albumin or cytochrome *c* was not bound by the specific binding site of the CRP ligand, PEA. A reason for this observation is that isoelectric point (p*I*) of albumin and cytochrome *c* are 4–4.5 and ~10.6, respectively. Therefore, above their p*I*s, they carry a net negative charge. Since the working pH was 12, albumin and cytochrome *c* supposed to be negatively charged. The adsorption onto the negatively charged PEA-GNP is unlikely. Moreover, cytochrome *c* could not catalyze the luminol–H_2O_2 as a peroxidase at the high alkaline condition (pH 12). Cytochrome *c* can be inactivated by the presence of an excess of hydrogen peroxide [27,44–46]. Finally, no enhancement of CL signal was observed upon adding PEA/CRP.

Interestingly, when CRP was added to the same solution, the CL intensity increased markedly. This observation further confirmed that neither albumin nor cytochrome *c* affected the luminol–H_2O_2/GNPs CL intensity, or perturbed the interaction of CRP with its ligand.

3.5. Comparative advantages of CL-based CRP estimation

Here we provide comparative features of CRP estimation using a GNP-based luminol–H_2O_2 CL method and a conventional ELISA. Our proposed methodology offered some unique advantages compared to ELISA in that it was more sensitive and accurate, required less time and no labeling, and was more inexpensive. The sandwich enzyme immunoassay type ELISA procedure was used for quantitative analysis of human CRP in standards and sera samples according to the standard protocol [47]. The LOD of ELISA was 310 fM, with a linear range of 310 fM–77 pM, and a linear regression equation, $y = 0.026x + 0.3087$ ($R = 0.9969$) (Fig. S4). On the other hand, the GNP-based luminol–H_2O_2 CL system, showed a 188 fM LOD in the case of CRP estimation. This means that the proposed methodology is 164 times more sensitive than the conventional ELISA method.

Additionally, Table 2 compares quantitative data generated using the GNP-based luminol–H_2O_2 CL system and conventional ELISA in terms of use for quantifying unknown CRP in human serum samples in a clinical setting. In all cases, the linear equations $y = 549.44x + 4307.9$ for anionic GNPs (Fig. 5A), $y = 1007.6x + 4613.6$ for cationic GNPs (Fig. 5B), and $y = 0.026x + 0.3087$ from the ELISA calibration curve (Fig. S4), were used to calculate the concentration of CRP in blood sera using observed CL intensities and optical densities (Tables S1 and S2). The blood sera required a 100-fold dilution for estimation of CRP concentration using the CL measurement method. The CRP concentration was then calculated by multiplying by the dilution factor. No significant differences were found between the results of the two methods using the Student's *t*-test, showing that the GNP-based CL detection system has a high accuracy at the 95% confidence level (Table 2) compared with the well-established ELISA technique. Moreover, there were no significant differences between the estimated CRP concentration using both the anionic and cationic GNPs (Table S3).

4. Conclusions

The effort of this study was to develop a methodology for the estimation of CRP-avoiding antibodies based on the catalytic activity of both the anionic and cationic GNPs within the luminol–H_2O_2 CL reaction. To achieve this goal, we synthesized both types of GNPs, applied both in a luminol–H_2O_2 CL system, and characterized the results using UV–vis spectroscopy, TEM, and a spectrofluorometer. The plasmon absorption spectra of GNPs were found to show a broad red shift in the presence of CRP with its ligand PEA, reflecting a change in GNP state from dispersed to aggregated, which greatly enhanced the luminol–H_2O_2 CL intensity. The concentration-dependent CL intensity changes assisted the measurement of CRP in serum samples using a luminometer and 96-well plate. The newly designed approach was used to estimate the CRP in serum of patients, and the values were compared with the well-established ELISA technique. The proposed approach offers label-free quantification of samples at concentrations up to 1.88 fM (=LOD) without using any antibody. The CL measuring procedure was similar to ELISA, but has no time limit. Moreover, our method demonstrated approximately a 164-fold greater LOD sensitivity and was much faster than ELISA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.02.001.

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