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Short communication

CdSe and ZnSe quantum dots capped with PEA for screening C-reactive protein in human serum

D. Gomes[a], M. Algarra[b,*], M.J. Diez de los Rios[c], M.M. Arrebola[c], M.E. Herrera-Gutiérrez[d], G. Seller-Pérez[d], J.C.G. Esteves da Silva[a]

[a] Centro de Investigação em Química, Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre 687, 4169-007 Porto, Portugal
[b] Centro de Geología, Departamento de Geociências, Ambiente e Ordenamento do Território do Porto, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre 687, 4169-007 Porto, Portugal
[c] Laboratorio de Urgencias, Servicio de Análisis Clínicos, Complejo Hospitalario Carlos Haya, Málaga, Spain
[d] Servicio de Cuidados Críticos y Urgencias, Complejo Hospitalario Carlos Haya, Málaga, Spain

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ABSTRACT

A fluorescence chemical sensor for C-reactive protein (CRP) was developed based on the selective interaction with CdSe and ZnSe quantum dots (QDs) coated with *O*-phosphorylethanolamine (PEA). Synthesis procedure and analytical parameters such as pH and ionic strength were studied. The decrease in the fluorescence emission intensity was explained due to the specific interaction of the QDs-PEA with CRP, and a correlation was observed between the quenching of the fluorescence and the concentration of CRP. The accuracy of the proposed method was 0.37% as RSD. The proposed method was applied to screen serum samples, and showed to be sensible at the C-reactive protein concentrations of risks levels.

1. Introduction

C-reactive protein (CRP) is a protein found in the blood, the levels of which rise in response to inflammation (an acute-phase protein). Its physiological role is to bind to phosphocholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system via the C1Q complex [1]. CRP is member of pentraxim family of proteins and synthesized by the liver [2] in response to factors released by fat cells (adipocytes) [3]. Determination of CRP levels can indicate a rapid diagnosis of infectious and inflammation in patients and has been given particular interest as a marker of inflammation associated with cardio vascular diseases (CVD) [4–9]. Indeed, taking into consideration the cardiovascular risk of patients, three CRP levels were proposed [4,7]: low risk (<1 mg L^{-1}); average risk (1–3 mg L^{-1}); and, high risk (>3 mg L^{-1}). Also, a 15 mg dL^{-1} CRP level was used as a cut-off point to differentiate between high-risk patients and patients who are prone to CVD in the next 6 month [10].

Screening CRP levels in blood can be done by several analytical techniques, such as enzyme immunoassay methods (EIA) [11,12], enzyme linked immuno-sorbent assay (ELISA) [13–16], immune-turbidimetry [17,18], rapid immuno-diffusion [19]; chromatographic technique as liquid chromatography with tandem mass spectrometry detection (LC/MS/MS) [20,21] or thin layer chromatography [22]; spectroscopy such as nephelometric [23,24], chemiluminescent [25] and fluorescence [26–30]. By means the competitive behavior of conjugated CRP assembled to magnetic [31] or gold nanoparticles have been used as analytical method to quantify the CRP levels in serum [32,33].

Fluorescent QDs can constitute the basis of new strategies to the development of methods to the detection of CRP. Semiconductor nanocrystals, such as CdS or CdSe, also referred to as quantum dots (QDs) show high fluorescence efficiency, robustness, and flexibility of functionalization with conjugating ligands for the selective nano-sensing of the analyte [34–36]. Studies revealed that the interactions between some substances and the surface of QDs would change their physical properties. Thus, expanding applications of QDs to develop sensitive and simple sensors for the detection of different analytes is a topic of current interest [37–41].

In this paper the synthesis of CdSe and ZnSe QDs coupled with *O*-phosphorylethanolamine, a specific synthetic ligand for CRP is described, together with its application in the determination of CRP

* Corresponding author. Tel.: +351 220 402 568; fax: +351 220 402 659.
E-mail address: magonzal@fc.up.pt (M. Algarra).

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in serum samples [32], as alternative to the immune assays techniques, which are very expensive and have tedious manipulation.

2. Experimental

2.1. Chemicals and reagents

Cadmium chloride (99.9%, $CdCl_2$), zinc nitrate ($Zn(NO_3)_2 \times 4H_2O$), selenium powder (Se, 99.99%), sodium borohydride ($NaBH_4$, ≥96%), mercaptosuccinic acid (MSA, >97%), mercaptoacetic acid (MAA, ≥99%), 3-mercaptopropinoic acid (MPA, 99%), C-reactive protein (CRP) from human plasma, and *O*-phosphorylethanolamine (PEA) were purchased from Sigma–Aldrich Química S.A. (Spain) and used without further purification. All solutions were prepared with deionized water with resistivity higher than 4 MΩ/cm.

The serum samples were obtained from patients from Carlos Haya Hospital, Málaga (Spain) according to their own assay procedure [42] and stored at −20 °C until prior analysis. The concentrations of the samples were purchased by means the C-Reactive Protein Extended Range (rCRP) method, an immune-turbidimetric assay. The analytical measurement range is: 0.5–250 mg L^{-1}. The analytical sensitivity is 0.5 mg L^{-1} (is the lowest concentration of CRP that can be distinguished from zero, the functional sensitivity (as RSD < 20%), for 1.1 mg L^{-1} was obtained RSD = 17.7%).

2.2. Synthesis of CdSe-PEA and ZnSe-PEA QDs

2.2.1. Synthesis of CdSe-PEA

CdSe QDs were prepared according the procedure carried out previously by our group with some modifications [43]. CdSe-PEA QDs were prepared using two different procedures, according the order of addition of PEA, and compared with the raw the CdSe QDs. 26 mg of $CdCl_2$ (0.142 mol) was dissolved in H_2O (50 mL) and left for 3 h followed by addition of 173 mg (1.15 mmol) of MSA. Same procedure was followed with other capping agents as MPA (100 μL) and MAA (80 μL). After 24 h, the pH was adjusted to 7 by means NaOH and 200 μL of the reduced selenium solution (NaSeH), obtained as previously reported (Supplementary information), was added to this solution, with continuously stirring for more 24 h, yielding an orange-red solution. The order of addition of PEA (40 mg) was checked and added as final reagent, and left for 24 h. This procedure supplies the highest fluorescence intensity, and chosen for further experiments.

2.2.2. Synthesis of ZnSe-PEA

Similar procedure was carried out to obtain ZnSe QDs, briefly: 7 mg of $Zn(NO_3)_2{\cdot}4H_2O$ were dissolved in H_2O (50 mL), by stirring for 3 h. Subsequently, MPA (17.6 μL) was added and left for 3 h. The same procedure was followed with MSA (10.8 mg) and MAA (7 μL). After 24 h, the pH was adjusted to 12 with triethylamine, and added the reduced Se (200 μL). Finally, PEA (14.3 mg) was added and left for other 24 h.

2.2.3. Order of addition of reagents

The order of addition of the capping agent and PEA were checked and it was observed that when PEA is added at the end of the synthesis procedure it resulted in higher fluorescence emission. This result can be explained due to those capping agents with thiol terminal groups, stabilizing the QDs surface, compared with the PEA molecule which has not susceptible functional groups to stabilizing them. Moreover, it was studied the photo-stability under UV light of the QDs functionalized with PEA and was stable (maintaining a constant emission) for 10 days (Supplementary information, Fig. SI-1). The synthesis procedure was monitored under heating conditions (60 °C), and showed a stable fluorescence emission between 15–36 h (Supplementary information, Fig. SI-2).

2.3. Fluorescence measurements

Fluorescence measurements were recorded with Horiba Jovin Yvon Fluoromax 4 TCSPC spectrophotometer using an excitation of 380 and 180 nm for CdSe-PEA and ZnSe-PEA QDs respectively, and an emission range of 300–740 nm, with an integration time of 0.1 s and a slit width of 5 nm for excitation and emission. Samples were analyzed in 1 cm path length quartz cuvette. Absorbance measurements for the measurements of quantum yields were made in a Hewlett-Packard HP8452A diode array spectrophotometer. The calibration curve for Rhodamine B was done in methanol with a refractive index (n_{st}) of 1.329. The integrated area under the fluorescence curves (excitation at 488 nm) was plotted versus the absorbance at 488 nm (after subtraction of the solvent absorbance) for different concentrations. The same procedure was repeated for the QD samples suspended deionized water ($n = 1.3749$). The excitation intensity and slit width (5 nm) were held constant for all measurements. The QY of the QDs was obtained from:

$$QY_{QD} = QY_{st}\left[\frac{(dI/dA)_{QD}}{(dI/dA)_{st}}\right]\left[\frac{n_{QD}^2}{n_{st}^2}\right]$$

where I is the area under the fluorescence curves and A is the corresponding absorbance [44].

2.4. Procedure for determination of CRP

Aliquots of CRP samples were placed in a 5 mL calibrated flask with 2 mL of the CdSe-PEA or ZnSe-PEA QDs solutions, and filled with $CaCl_2$ (0.01 M) at pH 7, to avoid the denaturalization of the CRP. Then the fluorescence spectra were recorded in the range of 300–740 nm, excited at 380 nm. The analysis of serum samples were done using the following procedure: aliquots of serum of concentrations between 10 and 50 mg L^{-1} were placed in 5 mL calibrated flasks with 2 mL of CdSe-PEA QDs and filled with $CaCl_2$ (0.1 M) aqueous solutions at pH 7, and the fluorescence measurements were done as described above.

3. Results and discussion

3.1. Synthesis and characterization of the PEA functionalized QDs

Fig. 1a shows the fluorescence spectra of CdSe-PEA QDs with the three capping agents tested, MSA, MAA and MPA. The analysis of this figure shows that the maximum of the fluorescence emission is located at 541, 573 and 590 nm, respectively, and the MSA capped QDs show the highest fluorescence intensity with a emission full width of half maximum (FWHM) 318 nm. This relatively high FWHM suggests that the CdSe-PEA QDs shows a high degree of size heterogeneity, probably because they are bound to different chemical environments. The ZnSe-PEA QDs were successfully synthesized, showing more fluorescence emission intensity than CdSe-PEA QDs. The fluorescence spectrum of ZnSe-PEA QDs (Fig. 1b) shows two prominent peaks at 411 and 465 nm respectively, in accordance with that obtained previously in the literature [45] – the FWHM is 295 nm.

The quantum yield (QY) obtained in water for CdSe-PEA and ZnSe-PEA QDs were 3.7 and 4.3% respectively (using Rhodamine B as reference). This small QY is due to the fact that the material has a major non-fluorescent component, the PEA that limits its fluorescence efficiency. The efficiency of the QY of CdSe and ZnSe QDs is relatively small due to the fact that it was calculated in water,

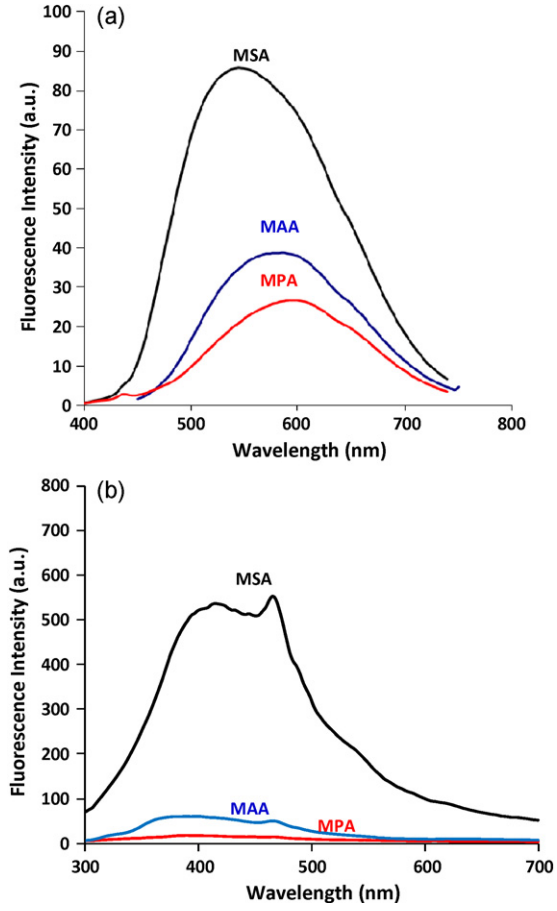

Fig. 1. Fluorescence spectra of (a) CdSe-PEA QDs at pH 7, λ_{ex} = 380 nm, and (b) ZnSe-PEA QDs at pH 12, λ_{ex} = 180 nm.

where they are highly soluble, and due to environmental effect some excitated state deactivation is observed.

3.2. Effect of pH and ionic strength

Fig. 2 shows the fluorescence intensity maximum of CdSe-PEA QDs as function of the pH. The analysis of this figure shows the maximum fluorescent intensity at pH 6–7, resulting compatible with serum samples analysis. Low pH values (< 5) provoke the rupture of the QDs, decreasing the emission; at pH > 8, the ionization of

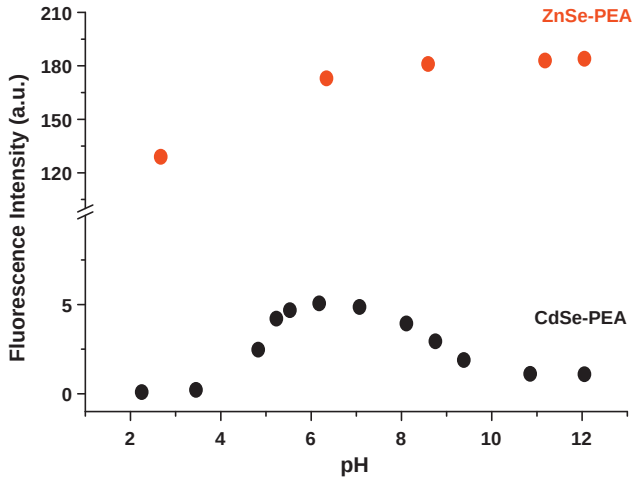

Fig. 2. Influence of pH over fluorescence of CdSe-PEA (a) and ZnSe-PEA (b) QDs.

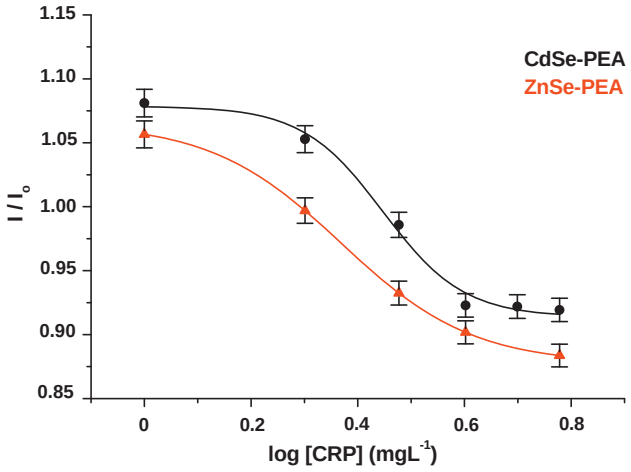

Fig. 3. Plot of the calibration curve in the proposed method conditions for analysis CRP at pH 7 by CdSe-PEA and ZnSe-PEA.

the carboxylic groups of MSA capping the CdSe-PEA QDs change the quantum confinement of the QDs [46]. In the case of ZnSe-PEA QDs the influence of pH has different profile, increase slowly until a constant fluorescence emission from acid to basic media.

The effect of alkaline metal ions (KCl) in the molar concentration range of 0–59.64 gL^{-1} (ionic strength effect) was studied and the results are shown in Supplementary information (Fig SI-3). For both QDs the fluorescence intensity shows a decrease up to 22.36 gL^{-1} and stays almost constant for higher salt concentration. The increase of the ionic strength provokes the quenching of the QDs fluorescence because it will affect the charge and composition of the QDs capping shell resulting in the unstabilization (possible destruction) of the QDs. Considering this ionic strength effect the CRP determinations were always done: pH 7 and ionic strength of 0.25 M ($CaCl_2$, 0.1 M).

3.3. Calibration curve

The effect of CRP (concentration range 0–8 mg L^{-1} in $CaCl_2$ (0.1 M) aqueous solutions at pH 7) on the CdSe-PEA and ZnSe-PEA QDs fluorescence is shown in Fig. 3. The variation in intensity with concentration of CRP showed a quenching of the fluorescence signal of both QDs when the concentration of CRP increases. The quenching of the QDs fluorescence is a consequence of the fact that PEA is a specific ligand of the CRP and, upon association, the stabilization effect of PEA on the QDs (discussed above in section 2.2.3) is no longer observed. However, apparently PEA is not completed removed from the QDs because the quenching is not directly proportional to the CRP concentration. Upon the binding with PEA the protein actively modify the QDs surface resulting in a non-radiative deactivation. According the synthesis procedure, mentioned above, the PEA molecules is added in the last step and the interaction must occur in the surface of the most external surfaces of it. The calibration graph fitted to the four parameter logistic nonlinear regression model at maximum of fluorescence emission (549 nm) are:

CdSe-PEA QDs-I/I_o

$$= \left(\frac{1.1358}{1 + 0.36233[\mathrm{CRP}]^{0.3461}}\right) + 0.83102 \; (\chi^2 = 1.37 \times 10^{-7})$$

ZnSe-PEA QDs-I/I_o

$$= \left(\frac{1.0785}{1 + 0.4471[\mathrm{CRP}]^{0.07541}}\right) + 0.91386 \; (\chi^2 = 8.94 \times 10^{-5})$$

Table 1
Results obtained with CdSe-PEA QDs in serum samples.[a]

CRP (mg/L)	CRP found (mg/L)	CRP labelled (mg/L)	CRP found (mg/L)	Error (%)
1	1.14 (0.73)	10	11.4	14.00
2	2.25 (1.92)	20	22.5	12.50
3	3.34 (2.43)	30	33.4	11.30
4	4.50 (2.91)	40	45.0	11.11

[a] Average and standard deviation for $n=3$ (under parenthesis and referring to the last significant digit) of three independent experiences.

where I and I_o are the fluorescence intensities of the QDs with and without CRP in the medium, respectively; a is the highest value obtained for I/I_o; b is a constant; [CRP] is concentration in mg L^{-1}; c (is the slope); and, d is the minimal detectable concentration (MDC) as the lowest concentration which results in an expected response less than the estimated one-sided α level ($\alpha=0.05$) lower confidence limit at zero concentration. The precision of the method obtained (as RSD) for 4 mgL^{-1} ($n=3$) was 0.37% for CdSe-PEA QDs and 2.19% for ZnSe-PEA QDs with 6 mgL^{-1} ($n=3$) respectively.

3.4. Analysis of serum samples

In order to assess the potential of the CdSe-PEA QDs based method for the analysis of CRP real serum samples of sick or recently operated patients were analysed. The results obtained using ZnSe-QDs were not acceptable because the estimated concentrations were much higher than the expected. Table 1 shows the results obtained for the analysis of several serum samples. The application of CdSe-PEA QDs showed the best results when the analytical procedure was applied in low range of CRP concentrations – comprised among 1–4 mgL^{-1}. The intrinsic fluorescence of the serum samples was minimized due to the dilution factor used in the serum samples preparation (at least ten times) and to the fact that the fluorescence of the QDs is relatively high.

4. Conclusions

In summary, both CdSe-PEA and ZnSe-PEA QDs were synthesized for the fluorescence determination of CRP by the presence of PEA, essential for the selective recognition of this protein. The pH and sequence of reagents addition were key factors for the successful synthesis of both QDs. The fluorescence intensity decreased in the presence of CRP and this quenching can be used to determine CRP in real serum samples. This method can be used to screen the presence of CRP levels in the concentration window that separates the normal and pathological values.

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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.2012.01.042.

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