INTRODUCTION

Urea, which is end product of protein metabolism and the main nitrogen component of urine, is an important biomarker monitored in blood and urine samples to diagnosis renal and liver diseases. Urea concentration above the normal level can be indication of renal failure, urinary tract obstruction and gastrointestinal bleeding (1,2). On the other hand low urea concentrations may be observed in hepatic failure, nephritic syndrome and cachexia. Therefore, it is essential to develop techniques for determination of urea in blood. Conventional spectroscopic methods are used for many years in clinical laboratories for determination of urea in blood samples (3 -5). However, these methods are time consuming due to sample pretreatment and unsuitable real-time determination of urea. Biosensors, based on urease enzyme are alternative methods for the determination of urea level.[6] To this end, a number of methods have been developed and reported such as potentiometry (7-9), voltammetry (10,11), conductometry (12-14), ion selective electrode (15), and spectrometry (16,17).

Quantum dots (QDs) are superior to organic dyes with their size tunable photonic properties, quantum yield and stability against photobleaching (18-21). Luminescence properties of quantum dots are highly sensitive to changes on their surface. Majority of the QD photoluminescent probes are based on the increasing or quenching of photoluminescence signal, which is caused by chemical or physical interaction. Thus the selective determination of analyte can be achieved via interaction with functionalized QD or non-functionalized QD (22-25)

QDs have been widely used as biosensors in biotechnology (24-27). Recently, a few applications of QDs for the determination of urea appeared in the literature (6,17,24). All of these studies are based on the pH change upon the reaction of urea and urease enzyme. Although, most of previous studies have been focused on QDs as fluorescence sensor, their long lifetime allows to use of phosphorescence mode has more advantages than fluorescence method such as the spectral interferences from biological matrices can be easily prevented in the phosphorescence mode (6,28,29).

This study describes a simple and reliable analytical method for the determination of urea in biological sample using MPA- CdTe QDs. The urea concentration was

determined in serum samples by monitoring the increase phosphorescence signal in the presence of urease enzyme.

EXPERIMENTAL

Materials

All the measurements were performed using analytical grade chemicals. Deionized water was used throughout the study. $CdCl_2$, H_2TeO_6, $NaBH_4$, NaOH, HCl and urease were obtained from Merck, Mercaptopropionic acid was obtained from Fluka. Trisodium citrate was obtained from Riedel de Haen and ethanol was obtained from Sigma-Aldrich. Dilute solutions of the quantum dot, urease and urea were prepared daily.

Apparatus

A Varian, Cary Eclipse Luminescence spectrometer equipped with xenon lamp was used for photoluminescence measurements. All instrumental parameters are controlled by instrument software. Automatic filter selection mode for both excitation and emission monochromator was used to avoid scattered light. Excitation and emission spectral band passes were 20 nm for both monochromator. The detector voltage was set to 800 V. An Orion 720 A model pH/Ionmeter was used for pH adjustments. Deionized water with 18.2 MΩ.cm was obtained from Millipore Simplicity water purification system. Nüve NF200 centrifuge and Nüve MK418 magnetic stirrer were used during the synthesis of the quantum dots. Unicam Mattson 1000 FTIR spectrometer was used to obtain IR spectra of the modified QDs to confirm surface modification. UV spectrum of QDs was obtained using Shimadzu UV-VIS spectrometer. Transmission electron microscopy (TEM) measurements were performed on a JEOL 2100 HRTEM instrument (JEOL Ltd., Tokyo, Japan). TEM samples were prepared by pipetting 10 µL of QD solution onto copper grids and allowed to stand for 10 min.

Synthesis of water-soluble MPA- CdTe QDs

MPA- CdTe QDs were synthesized method modified from Yuan J et al (30). The method, 25 mL 0.64 mM $CdCl_2$ solution and 0.10 g of trisodium citrate was transferred into a single-necked flask. Then, 100 µL, 11.5 M MPA and 0.01 mmol Te (IV) were added respectively, with continuous stirring. The color of the solution becomes bright yellow with the addition of 50 mg of $NaBH_4$, and it is heated to 90 °C

for 1 hour with continuous stirring. After cooling, QDs were precipitated with ethanol, centrifuged and dried in vacuum. Each batch was end up 80-100 mg dry powder QD and in order to have a constant QD concentration, a 75 mg portion of the QDs powder was re-dissolved in water and diluted to 25 mL volume. At this stage, pH of the quantum dot solution was adjusted to 11.4 and heated to 96-100 °C for different period of time. An increase in the particle size as well as the fluorescence emission at longer wavelength was observed.

Procedure for urea determination

A 10.0 mL of 3.0 mg mL^{-1} MPA-CdTe and 10 mL of 5 units mL^{-1} urease solutions were placed into the beaker and pH was adjusted to 2.5 with 0.01 M HCl. The solution was transferred to a 25 mL volumetric flask and diluted to the volume with deionized water. A series of standard solutions were prepared by transferring 1.0 mL of the mixture solution into a test tube and then, various volume of urea standard solution or 0.10 mL of serum samples were added. The volume was completed to 5.0 mL with deionized water. The solutions were mixed and allowed to stand for 10 minutes at laboratory temperatures. The measurements were performed using phosphorescence mode with a 0.1 ms delay time and 3 ms gate time. Excitation wavelength was 300 nm and spectral band passes were 20 nm for both excitation and emission monochromators.

Samples

Human serum samples were collected from healthy volunteers. The samples were diluted 50 times with deionized water adjusted pH 2 with 0.01 M HCl before the measurement procedure. The same samples were analyzed for urea using standard methods used in clinical laboratory in order to test the accuracy of the proposed method.

RESULTS AND DISCUSSION

Characterization of MPA-CdTe QDs

The quantum dots were characterized using fluorescence, UV-VIS, Infrared spectroscopy and TEM images. After synthesis procedure (without thermal pretreatment) the quantum dots have fluorescence emission maximum at 505-510 nm with a full width at half maximum (FWHM) about 35 nm and almost no

phosphorescence signal as shown in Figure 1. On the other hand, when this QD was heated to 90-100 °C (pH 11.4) for different period of time an increase in the particle size as well as fluorescence and phosphorescence emission intensity was observed. Therefore the heating period of three hours, which provides intense phosphorescence signal at longer wavelength, was selected to avoid fluorescence background emission from the biological sample. The phosphorescence spectra of MPA capped CdTe heated at different period of times are shown in Figure1.

The diameter of CdTe QD heated for 180 min (Figure1) was calculated using the equation given below (27).

$D = (9.8127 \times 10^{-7})\,\lambda^3 - (1.7147 \times 10^{-3})\,\lambda^2 + (1.0064)\lambda - 194.84$

D is the diameter of the nanocrystals (nm); λ is the wavelength corresponding to absorbance maximum determined as 560 nm from the UV VIS spectrum as shown Figure 2a Calculations show that the diameter of the MPA-CdTe QDs is 3.34 nm. The TEM image of MPA-capped CdTe QDs was shown in Figure 2b.

FT-IR spectroscopy was used to confirm the modification of CdTe QDs with MPA molecules. The spectra of free MPA and MPA-CdTe are given in Figure 3A and B. The two bands at 2666 and 2570 cm^{-1} which is attributed to the hydrogen bonding between acid and thiol

groups were disappeared in IR spectra of MPA capped-CdTe because of the MPA is attached to QD through S-atoms. The small peaks appeared at 2927, 2945 and 2854 cm^{-1} was attributed to the asymmetric and symmetric C-H stretching of methylene groups. The appearance of an intense peak at 1570 cm^{-1} can be attributed to asymmetric stretching of carboxylic acid.

The effect of pH and concentration of urease on phosphorescence intensity of MPA capped CdTe QDs

pH is one of the important parameter that affect the photoluminescence intensity of the QDs. Therefore, the effect of the solution pH on the signal intensity of QD was studied using 0.5 mL of 0.04 M Britton–Robinson buffer between 2.5-8.0. It was observed that the phosphorescence signal was increasing linearly as the pH was increased from 2.5 to 5.0, and decreased between the pH 5.0-8.0. Therefore, the pH between 2.5-5.0 was selected for the determination of urea in the presence of QD and urease enzyme. Interestingly, influence of pH on the fluorescence signal was

different than the signal measured in phosphorescence mode. The effect of pH on photoluminescence intensity of MPA - CdTe QDs was shown Figure 4.

The effect of urease enzyme concentration was studied between 1-7.5 units/mL in the presence of 0.24 mg L^{-1} MPA-CdTe and 0.07 mM urea. The maximum signal enhancement was observed when the urease enzyme concentration was 5 units/mL and used throughout the experiments.

Determination of urea

Determination of urea is based on the production of ammonia in the presence of urease.

$$CO(NH_2)_2 + 3H_2O \xrightarrow{\text{urease}} 2\,NH_4^+ + OH^- + HCO_3^-$$

The pH of medium is increased depending on degradation of urea by urease enzyme. Consequently, phosphorescence signal was increasing throughout the pH 2.5-5.0 range with increasing urea concentration. In the optimum conditions (0.24 mg mL^{-1} CdTe-MPA, 5 units mL^{-1} urease and pH 2.5), the calibration was constructed by plotting $I-I_0$ versus urea concentration.(I_0: phosphorescence signal of CdTe-MPA I: phosphorescence signal of CdTe-MPA+ 0.0016-0.16 mM urea) The linearity in phosphorescence signal was observed between 0.016 -0.16 mM urea concentrations. Phosphorescence signal with increasing urea concentration is shown Figure 5b. Although the fluorescence signal was increasing with urea concentration, the dynamic range was relatively narrow as shown in Figure 5b. The calibration curve based on phosphorescence signal is given in Figure 6.

The lifetime software of the instrument was used to obtain decay curve for the phosphorescence emission and the data was used to construct log intensity versus time graph. Lifetime of the QD was calculated using the -1/slope of this linear line and found as 21.5 µs. (Figure 7)

The proposed method was compared with the methods in the literature. Limit of detection for the proposed method was lower or comparable with the methods such as fluorescence, amperometry and potentiometry (Table 1). Analytical performance data of the method used for urea determination are given Table 2. On the other hand the proposed method is relatively simple and free from the interferences from the biological matrix since the phosphorescence signal was used.

Determination of urea in human serum

Serum samples obtained from university laboratory were analyzed for urea using the proposed method. In order to test the accuracy of the proposed method, the same samples were analyzed in private clinical laboratory and the results were shown in Table 3. The precision in terms of percent RSD, for three parallel determinations was less than 6.7 % and the urea concentrations were in consistent with those reported .

CONCLUSION

It was shown that the water-soluble and phosphorescent MPA - CdTe QDs can be used for the determination of urea in human blood serum samples. The proposed method based on enzymatic degradation of urea by urease enzyme.In addition, the use of phosphorescence prevents interferences such as scattering and autofluorescence from the sample matrix. Compared to conventional room temperature phosphorimetric methods, phosphorescent QDs provides simpler methodology since no additional chemicals such as heavy atom and oxygen removal process are necessary. The results show that the proposed method is accurate, selective, rapid and simple for urea determination in serum samples and can be applied to other biological samples.

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