A Membrane-Based Disposable Well-Plate for Cyanide Detection Incorporating a Fluorescent Chitosan-CdTe Quantum Dot

Sumate Pengpumkiat, Yuanyuan Wu, Saichon Sumantakul and Vincent T. Remcho*

Department of Chemistry, Oregon State University, 153 Gilbert Hall, Corvallis, OR, USA, 97331

*Corresponding Author: Vincent T. Remcho  Email: vince.remcho@oregonstate.edu

Tel.: +1 541 737 8181. Fax: +1 541 737 2062

To whom correspondence should be addressed

Email: vince.remcho@oregonstate.edu
Abstract

A novel approach to building a membrane-based disposable well-plate, here applied to cyanide detection, is described. Chitosan encapsulated CdTe quantum dots with a maximum emission at 520 nm (CS-QD520) were used as fluorophores. The CS-QD520 nanoparticle was specifically quenched by copper (II), and the quenched CS-QD520 (Cu-CS-QD520) was deposited onto a glass microfiber filter (GF/B). Subsequent introduction of cyanide ion resulted in fluorescence recovery. The “signal-ON” fluorescence linearly correlated to cyanide concentrations in the range of 38.7 to 200 µM with a limit of detection of 11.6 µM. The assay was incorporated into a membrane-based well-plate format to enhance sample throughput. A three-layer paper/glass microfiber well plate design was cut using a laser cutter and assembled using a polycaprolactone (PCL) as a bonding agent in a low-cost laminator. The experimental conditions were optimized and applied to detect cyanide in drinking water with rapid, high-throughput, low-cost analysis.

Keywords: Well-plate, Cyanide Detection, CdTe Quantum Dot
Introduction

Free cyanide is severely toxic to humans. Major uses of cyanide are from some metal mining processes, photographic developing, chemical industries, combustion of plastic and vinyl as well as cyanide-containing pesticides. Cyanide can enter the human body via respiration, ingestion, and absorption through the skin. Cyanide in the blood exists as HCN (a weak acid with $pK_a = 9.21$) and freely crosses all biological membranes and binds to heme ($Fe^{3+}$) in the cytochrome a-a3 complex. It readily works as a competitive inhibitor causing decoupling of oxidative phosphorylation. This action prevents the cell from utilizing oxygen leading to the rapid onset of cellular hypoxia – flushing, headache, tachypnea, respiratory depression, complete heart block and death. The US Environmental Protection Agency (US EPA) has set a maximum contaminant level (MCL) for cyanide in drinking water at 7.7 µM.

The US EPA standard analytical method for detection of cyanide is spectrophotometric. Chloramine-T in a pyridine-barbituric acid solution is used to react with free cyanide to form a colored complex measured at 578 nm. Several conventional detection techniques for cyanide have recently been reviewed including UV-Visible spectroscopy, capillary electrophoresis, potentiometry, voltammetry, ion chromatography with pulsed amperometric detection (IC-PAD), and gas chromatography with mass spectrometric detection (GC/MS). These analytical methods are time-consuming and require both a laboratory setting and a skilled analyst.

Another approach for measurement of cyanide is a chemodosimeter, which uses chromogenic or fluorogenic probes that change color when they react with cyanide ions. The selectivity of detection is based on cyanide’s property as an exceptional nucleophile (stronger than commonly encountered ions) to functional groups presented in the probes. The selective chemical reactions employed in these sensors include carbon-carbon bond formation, proton transfer, electron transfer, and aggregation-induced emission (AIE). Although the detection limits for these approaches can be as low as tens of nM, the assays are generally performed in a mixed aqueous/organic solution, and the chromogenic or fluorogenic probes are derived from multistep organic syntheses conducted in organic solvents. Recently, bioluminescence assay with Escherichia coli has been applied for cyanide detection. Cyanide transports in the cell membrane, and binds to
Fe$^{3+}$ in cytochrome $c$ oxidase. This process produces excess amount of H$_2$O$_2$, which trigger the sensor protein to generate bioluminescence. The luminescence signal corresponds to cyanide concentration.$^{24}$

Semiconductor nanocrystals or quantum dots (QDs) have superior optical properties relative to the majority of organic fluorophores, namely high photostability, highly tunable emission wavelength, large Stokes shift, broad absorption spectra and narrow emission peaks. The fluorescent sensing application has been reviewed for toxic metals and biological molecules.$^{25}$ Others have used tri-$n$-octyl phosphine oxide (TOPO)-coated CdSe QDs$^{26}$ and mercaptosuccinic acid-capped CdTe QDs$^{27}$ for cyanide determination in water samples by prior quenching of their fluorescence with copper ions. The introduction of cyanide caused the dissociation of copper from the QDs surface resulting in fluorescence recovery. The modulation of QDs and copper as a sensor enables a simple approach for cyanide detection with a very low detection limit.

Due to the fact that cyanide is extremely toxic, causing acute cytotoxic hypoxia, rapid screening or point-of-need testing would be invaluable for quick detection in the field. The Office of the Chief Medical Examiner of New York City uses a commercially available paper test, Cyantesmo® (Macherey-Nagel, Dueren, Germany) to qualitatively screen for the presence of cyanide in aqueous solution in the lab.$^{28}$ The Cyantesmo® kit has been validated in water samples$^{29}$ (9.6 – 1154 $\mu$M), blood$^{30}$ (7.7 – 384 $\mu$M), and plant material$^{31}$ (38 – 1923 $\mu$M) at clinically relevant concentrations. In water samples, a cyanide concentration greater than 38 $\mu$M can be semi-quantitatively detected using this assay in 5 min. However, more than 2 h are required for developing color in response to concentrations lower than 29 $\mu$M, limiting the ability of this assay to rapidly detect low but clinically relevant concentrations of cyanide.

Paper microzone plates are a class of microfluidic paper-based analytical devices ($\mu$PADs) containing hydrophilic/hydrophobic barriers for fluid handling. This type of diagnostic device, related to conventional 96- and 384-plastic well plates, has been produced by the Whitesides group using a photolithographic fabrication technique.$^{32}$ Our approach to production of a membrane-based well plate follows a simpler lower-cost approach and uses a unique materials set that is optimized for this assay. We employ a biodegradable hydrophobic polymer, polycaprolactone (PCL), to generate
hydrophobic barriers on either paper or glass microfiber media, along with a low-cost laminator to assemble multi-layer devices. Assay “spots” containing all necessary reagents in dry form comprise each individual well and allow for parallel analysis of multiple samples and standards. Measurements are independently performed in this high-throughput format.

Here, we demonstrate a membrane-based disposable well-plate for cyanide detection using a unique copper quenched chitosan encapsulated CdTe quantum dot (Cu-CS-QD520) complex as a sensor. The sensing mechanism for the detection is shown in Fig. 1.

![Fig. 1]

Our well plate design used chitosan encapsulated CdTe quantum dots with a maximum emission at 520 nm (CS-QD520) as fluorophores, which were specifically quenched by copper (Cu2+) (Cu-CS-QD520). The quenching mechanism was mentioned in our previous report.\(^3\) The Cu-CS-QD520 was used as a sensor probe for cyanide detection. Upon cyanide introduction, the fluorescence signal was recovered due to the formation of a copper-cyanide complex \([\text{Cu(CN)}_x\text{]}}\(^{1-x}\), thus freeing the CS-QD520. The “signal-ON” fluorescence was graphed against cyanide concentrations in the relevant concentration range.

The fluorescence assay was preconcentrated on the surface of a PCL-patterned glass microfiber (GF/B) active layer (comprising the wells themselves) laminated between black paper upper and lower layers to yield a conventionally-sized disposable microtiter plate, providing enhanced signal relative to solution-based assays due to the higher surface-to-volume ratios of the assay in this format. Each well on the well plate is capable of performing an independent assay, thus the plate in its entirety is suitable for the analysis of up to 96 samples or standards simultaneously. The membrane-based well plate format incorporates disposable µPADs into an existing and ubiquitous analytical instrument, the microplate reader. The platform enabled rapid, inexpensive testing, high sample throughput and reliable quantitative results with excellent cyanide specificity.

**Experimental**

**Materials**

All of the materials and chemicals used for this study are listed in the supporting information (SI).
Synthesis of CdTe quantum dots (QD520)

3-Mercaptopropionic-acid-capped CdTe quantum dots (QD520) were synthesized in aqueous solution as previously described with some modifications. Two mmol anhydrous CdCl₂ (0.3666 g) and 4.8 mmol 3-mercaptopropionic acid (418 µL) were mixed in 200 mL of Milli-Q water. The pH of the solution was adjusted to 11 using 1.0 M NaOH. The mixture was purged with N₂ gas for 30 min to eliminate oxygen from solution. The NaHTe solution was prepared separately by mixing 0.5 mmol tellurium powder (63.8 mg) and 5 mmol NaBH₄ (0.1891 g) in 5 mL Milli-Q water. The NaHTe precursor was added to the cadmium precursor solution and the mixture was refluxed at 95-100 °C for 3 h under N₂ atmosphere. The final molar ratio of Cd²⁺/MPA/NaHTe was 1:2.4:0.25, respectively. The average diameter and concentration of QD520 were calculated as 1.76 nm and 81.7 µM, respectively. The calculation was shown in Fig. S1.

Synthesis of chitosan encapsulated CdTe quantum dots (CS-QD520)

Chitosan stock solution (1% w/v) was prepared by dissolving 1 g chitosan in 100 mL of 1% (v/v) acetic acid. The stock solution was subsequently diluted to 0.01% (w/v) using 0.1% (v/v) acetic acid. As prepared QD520 solution (22 mL) was gradually added in 100 mL of 0.01% (w/v) chitosan solution with stirring, followed by addition of 1 mL EDC (10 mg mL⁻¹)/NHS (5 mg mL⁻¹) solution. The mixture was further stirred overnight at room temperature to form covalent bonds between carboxylic acids on quantum dot surface and amino group on chitosan. The CS-QD520 was washed 3 times with Milli-Q water by centrifugation. In the final step, the precipitate was re-dispersed and stored in 22 mL (the same volume as QD520) phosphate buffer 10 mM, pH 7.4. The concentration of CS-QD520 would be 81.7 µM derived from QD520 concentration. The mass/volume concentration is 3.76 mg mL⁻¹.

Cyanide probe construction – Cu²⁺ quenching to produce Cu-CS-QD520

The stock CS-QD520 solution was diluted ten-fold with Tris-HCl buffer (10 mM, pH 7). An appropriate volume of copper (Cu²⁺, CuSO₄) standard (100 mg/L) was added to the diluted CS-QD520 solution with stirring to quench the fluorescence signal. The quenching reaction was continued for 3 h. Twenty microliters of the quenched solution (Cu-CS-QD520) was dropcasted onto
individual glass microfiber filters, grade GF/B (6.8 mm diameter), cut using a hole punch. The sensor dot thus prepared was dried overnight at 40°C in a vacuum oven prior to placement in the pre-punched “sandwich” of upper and lower well plate components.

**Membrane-based disposable well-plate and plastic plate holder fabrication**

The membrane-based well plate was designed and drawn to match the dimensions of a standard 96-well plate using SolidWorks® 2013 (Dassault Systèmes, Waltham, MA, USA) as shown in Fig. 2. The design contained three layers, the middle layer being the “active” or assay layer, consisting of the glass microfiber discs housed in-plane in a pre-punched array format. The bottom and pre-punched middle layers were composed of black poster paper (0.45 mm thickness). The top layer was composed of black filter paper (0.15 mm thickness). Each well in the middle layer had a diameter of 6.8 mm to match with the GF/B sensor dot. The top layer contained wells with a diameter of 5.4 mm, which was smaller than the wells on the middle layer. This locked the GF/B sensor dots in place in the middle layer. The poster paper and the filter paper were cut using a commercial laser cutter (50W CO₂ laser cutter, Universal Laser Systems Inc., Scottsdale, AZ, USA). A low-cost thermal laminator (Scotch, model TL902A) was used to press and bond all layers together using the pre-deposited, dried polycaprolactone (5% w/v in toluene) as the bonding agent. Two layers were laminated at a time: a top/middle layer was laminated and laid upside down, the GF/B sensor dots were placed in the wells, and then the bottom layer was added and the entire assembly was once again run through the laminator. PCL thus served as the bonding agent and as a hydrophobic barrier between each individual well to define and protect each individual assay on the plate. The disposable well plate was stored at room temperature in a vacuum-sealed bag until used. The membrane-based well plate was designed to be placed in a plate holder, which was compatible with the tray of any standard plate reader.

[Fig. 2]

The plate holder (Fig. 3A) was drawn using SolidWorks® 2013 and printed with a 3D printer (Ultimaker2, Dynamism, Inc., Chicago, IL, USA) using polylactic acid (PLA) filament. The full plate was fit inside the plate holder for use in the plate reader (Fig. 3B).
Determination of cyanide ion on a disposable well plate

Disposable membrane-based well-plates were used in combination with a plastic holder as described above to facilitate use in a standard fluorescence plate reader. Twenty microliter aliquots of cyanide standards (KCN) or samples were applied directly to the membrane-based well plate. The liquid sample sorbed into the GF/B substrate and interacted with the Cu-CS-QD520 that had been pre-deposited onto the top surface of the glass membrane filter active layer. The fluorescence signal was read at 520 nm with the excitation wavelength at 350 nm.

Results and discussion

Characterization of CdTe (QD520) and chitosan encapsulated CdTe (CS-QD520) quantum dots

The CS-QD520 was assembled in aqueous solution via electrostatic attraction between the positively charged amino functional group on chitosan and the negatively charged 3-mercaptopropionic acid (MPA) group on the QD520 surface. The chitosan and QD520 were covalently linked through amide bond formation by carbodiimide chemistry. The emission spectra at the excitation wavelength 350 nm for the QD520 and CS-QD520 thus prepared, shown in Fig. S2 (A), indicated that fluorescence intensity of the CS-QD520 was 3.5 times greater than that of the original QD520 at the same mass/volume concentration. This enhancement phenomenon may result from the intrinsic properties of chitosan. Numerous amino and hydroxyl functional groups on the biopolymer are good capping agents, which helped stabilize the QD surface. The morphology of CS-QD520 was studied by TEM (FEI Titan with ChemiSTEM, FEI Co., Hillsboro, OR, USA) as shown in Fig. S2 (B), which revealed the irregular shape of the nanoparticles with a particle size of 10 – 25 nm. Elemental analysis in the ChemiSTEM mode (Fig. S2 (C)) confirmed the presence of cadmium and tellurium in the CS-QD520 dots.

Chitosan was incorporated into the QD520 in order to facilitate QD immobilization on the well plates. Chitosan is a biocompatible and biodegradable polymer, which has been integrated with nanomaterials or microfluidic chips in the biomedical field. Chitosan contains a preponderance of amino groups on the side chain which are readily available for bioconjugation, exhibiting a positive
charge at acidic to neutral pH \( (pK_a \text{ of D-glucosamine unit} = 6.5 - 7.0) \)\(^4^3\) When CS-QD520 was applied to the glass microfiber filter containing silanol functionalities, the CS-QD520 nanoparticle electrostatically adsorbed strongly to the top surface of the substrate (in comparison, when QD520 alone was applied it was thoroughly absorbed into the substrate). We evaluated the extent of retention of the CS-QD520 on the glass microfiber medium as shown in the Supporting Information, Fig. S3. The CS-QD520 was well retained on the top surface of the substrate even after flushing with 10 mL of phosphate buffer (pH 7). The CS-QD520 remained in a tight narrow band on the top surface of the GF/B, whereas the original QD520 leached off from the substrate. Having a low fluorescence background and loading volume capacity, the glass microfiber filter enabled the retention of CS-QD520 on its top surface and also improved the homogeneity of the fluorescence signal, enhancing its performance.

**Detection mechanism and selectivity**

The cyanide sensor probe employed here provides a “signal-ON” fluorescence increase arising from complexation of the target analyte, cyanide, with a copper quenching agent\(^4^4\) to yield a highly stable \([\text{Cu(CN)}_x]^{1-x}\) product.\(^2^7,4^5\) This phenomenon leads to fluorescence recovery of the CS-QD520 that is directly proportional to the cyanide concentration in the sample. The “signal-ON” sensor largely eliminates the possibility of false positives (owing to the high selectivity of the complexation process) and enables higher sensitivity than is achievable with a “signal-OFF” sensor.\(^2^7\)

The selectivity of the assay on the membrane-based well plate was evaluated using a battery of common anions and cations, including \(\text{F}^-, \text{Cl}^-, \text{I}^-, \text{SCN}^-, \text{CH}_3\text{COO}^-, \text{NO}_3^-, \text{C}_2\text{O}_4^{2-}, \text{CO}_3^{2-}, \text{SO}_4^{2-}, \text{S}^{2-}, \text{Mg}^{2+}, \text{Al}^{3+}, \text{Mn}^{2+}, \text{Zn}^{2+}, \text{Fe}^{2+}, \text{Cd}^{2+}, \text{Ni}^{2+}, \text{Co}^{2+}, \text{Hg}^{2+}, \text{and Pb}^{2+}\) at a concentration of 1000 \(\mu\text{M}\). The fluorescence signals for the assay as conducted using these samples was monitored and compared with the fluorescence obtained from cyanide at 100 \(\mu\text{M}\). The results shown in Fig. 4 indicate that none of the species evaluated generated a false positive, hence, the sensor probe was highly selective toward cyanide.

Please note that, as is the case for the competing EPA standard method (method 335.4), the detection method described here was designed to determine free cyanide in a solution. Thus, if certain
interferents (specifically chlorine and/or sulphide, which can degrade cyanide into other forms) are present in solution, the sensor probe will indicate a falsely low concentration or a false negative. As for heavy metals coexistence in a solution, there will be metal-cyanide complexes instead, which are much less toxic and more stable than free cyanide.46–49

[Fig. 4]

**Optimization of the cyanide assay on the membrane-based well plate**

The highly selective assay in the membrane-based format presented several advantages over a solution-based assay. One benefit was that membrane-based well plates offered higher sensitivity (slope of calibration) than that of solution-based formats. The experimental data provided in Fig. S4 clearly show that the dried assay in the well plate format generates ~20-fold greater sensitivity (slope of calibration) than the same assay performed in solution. This may be attributed to the high surface-area-to-volume ratio of the GF/B medium used as a supporting material. Cu-CS-QD520 sensors were filtered and preconcentrated on the top surface of GF/B, further enhancing signal intensity.

In order to maximize the sensitivity of the assay we optimized the assay chemistry, including: CS-QD520 concentration, amount of Cu$^{2+}$ quencher, and post-sample-application reaction time. First, CS-QD520 concentration was varied from 4.08 – 20.43 µM. The calibration graphs in Fig. S5 (A) showed that the calibration slopes dramatically increased for CS-QD520 concentrations from 4.08 µM to 8.17 µM and then plateaued. The optimized CS-QD520 concentration was then set at 8.17 µM for all ensuing work. Second, the amount of Cu$^{2+}$ provided to modulate the fluorescence signal was optimized. If there is free Cu$^{2+}$ in the assay, it will first form a complex with the added cyanide and no fluorescence is generated. On the contrary, if there is less Cu$^{2+}$ than needed quantitatively for the assay, a high fluorescence background will be obtained, which then adversely impacts the detection limit. The optimum amount of Cu$^{2+}$ (100 mg/L) for CS-QD520 quenching was found to be 40 µL per 1 mL of 8.17 µM CS-QD520 by experiment (Fig. S5 (B)). Third, the reaction time for fluorescence recovery was monitored, shown in Fig. S5 (C). After the introduction of cyanide, fluorescence slowly regenerated owing to the release of copper from Cu-CS-QD520 to create free CS-QD520. Longer reaction times were found to lead to greater assay sensitivity; however, it is desirable to minimize the required reaction time while also maintaining high sensitivity (to facilitate practical application of the
The optimum reaction time was found to be 30 min, as this produced acceptable sensitivity with low standard deviation in multiple replicate analyses.

**Quantitative cyanide analysis**

A calibration curve for cyanide detection in water samples was established using the optimized conditions described above. Quantitative calibration data was collected both same-day and between-days (4 days; n = 12). A linear calibration plot in the range of 38.7 - 200 µM was generated from the entire data pool; \( y = 27.441x + 1398 \) (\( R^2 = 0.997 \)) (Fig. 5).

![Fig. 5](image)

Limit of detection (LOD) and limit of quantification (LOQ) were determined using the International Union of Pure and Applied Chemistry (IUPAC) definitions. LOD and LOQ were 11.6 µM and 38.7 µM, respectively. The LOD presented here by membrane-based disposable well-plate was comparable to the commercially available, “Cyantesmo kit” (9.6 – 1154 µM) with quantitative results, while the Cyantesmo kit is semi-quantitative.

**Practical sample analysis**

Practical cyanide analysis was performed using drinking water as a matrix. Drinking water was obtained from a local store, adjusted to pH >12 with 50% NaOH and analysed using the assay. As expected, cyanide was not detected in the drinking water. The accuracy and precision of the detection system were assessed by measuring recovery at three standardized cyanide concentration levels spiked into drinking water: low (50 µM), medium (100 µM), and high (200 µM), all in the linear range of the assay (Table 1). At low cyanide concentration (50 µM), the %RSD was larger than that of high cyanide concentration (200 µM). The membrane-based disposable well-plate demonstrated high accuracy with acceptable recovery (the recovery levels recommended by the Association of Official Analytical Chemists (AOAC) International are in the range of 75-120% at the 1 ppm concentration level). The lower recovery of cyanide at 50 µM may be a result of trace concentrations of metal ions in the drinking water samples, which would deplete the fortified cyanide standard leading to decreased signal intensity.

Table 1. Recovery of cyanide at different concentrations in drinking water (n=10)
Sample | Added CN⁻ (µM) | Measured CN⁻ (µM ± SD) | Recovery (% ± SD) | RSD (%) |
--- | --- | --- | --- | --- |
1 | 50 | 37.64 ± 5.33 | 75.28 ± 10.66 | 14.16 |
2 | 100 | 82.72 ± 8.75 | 82.72 ± 8.75 | 10.57 |
3 | 200 | 198.25 ± 12.44 | 99.13 ± 6.22 | 6.27 |

Conclusions

We have developed a rapid cyanide detection system on a membrane-based well plate using a novel fabrication technique. Polycaprolactone was used to assemble hydrophobic barriers on each individual well/assay and also used as an adhesive for assembling plate layers. The modulation of Cu²⁺ on CS-QD520 nanoparticles on the well plate enabled “signal-ON” detection for the presence of cyanide in a range of 0-200 µM. The membrane-based well plate for cyanide detection was validated using spiked drinking water samples, and showed a high precision and accuracy. The membrane-based disposable well-plate offers a simple, rapid, high-throughput, low-cost approach to cyanide detection in water samples in the relevant range of concentration, providing comparable sensitivity to a commercially available assay with the added benefits of quantitative (rather than qualitative to semi-quantitative) detection, increased throughput, and reduced cost.

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Figure Captions

Fig. 1 Schematic diagram of a membrane-based well plate for cyanide detection based on Cu-CS-QD520 complex.

Fig. 2 A membrane-based well plate resembling a standard plastic well plate designed for use in a conventional plate reader: (A) Layered-view, (B) A photograph of membrane-based well plate.

Fig. 3 (A) A plate holder for the membrane-based well plate. Base and Top-cover were designed using SolidWorks® 2013 and printed using an Ultimaker 2 3D printer. (B) A photograph of plate holder obtained from 3D printer.

Fig. 4 Selectivity of the assay on membrane-based well plates: (A) A battery of potential competing anions at 1000 µM, compared to cyanide at 100 µM; (B) A battery of potential competing cations at 1000 µM, compared to cyanide at 100 µM. (n=3)

Fig. 5 Endpoint calibration graph derived from membrane-based well plates for cyanide detection; [optimum condition: CS-QD520 = 8.17 µM, amount of Cu^{2+} (100 mg/L) = 40 µL/ 1 mL of 8.17 µM CS-QD520]
Membrane-based well plate

Fig. 1
Fig. 2

A Layered view

- Top layer: Black filter paper Ø 5.4 mm
- GF78 sensor dots Ø 6.8 mm
- Middle layer: Black paper Ø 6.8 mm
- Bottom layer: Black paper

B Top view

- 6.86 mm
- 114.15 mm
- 72.01 mm
- 119 mm
- 119.00 mm
- 77.76 mm

Dimensions in millimeters.
Fig. 3
Fig. 4
Fig. 5

A

\[ y = 27.441x + 1398.5 \]
\[ R^2 = 0.997 \]

Relative Fluorescence Unit

Cyanide [\mu M]

B

Cyanide [\mu M] 0 10 20 30 50 70 100 150 200 250 300
Row A
Row B
Row C
Graphical Index

Membrane-based well plate