Functionalized carbon quantum dots with dopamine for tyrosinase activity analysis

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Keywords: Tyrosinase; Carbon quantum dots; Fluorescence assay; Photo-induced electron transfer

Abstract
Tyrosinase (TYR) is a key enzyme in melanin biosynthesis and its activity is an important biomarker for dermatological disorders, such as vitiligo, melanoma and actinic damages. Sensitive assay for TYR activity is significant for basic and clinical research. In this work, a facile fluorescent assay for TYR activity based on dopamine functionalized carbon quantum dots (CQDs-Dopa) has been developed. Dopamine (Dopa) was covalently bond to CQDs through a simple one-pot hydrothermal method, and the prepared CQDs-Dopa exhibited a fluorescence emission at 499 nm under exciting wavelength at 310 nm with a quantum yield of approximately 2.1%. When TYR was mixed with CQDs-Dopa, the dopamine moiety in CQDs-Dopa conjugate was oxidized to O-dopaquinone, and an intra-particle photo-induced electron transfer (PET) process consequently occurred between CQDs and O-dopaquinone to quench the fluorescence of CQDs-Dopa. TYR activity can be determined based on the fluorescence quenching degree of CQDs-Dopa. This assay covered two broad linear ranges: 44.4–711.1 U L⁻¹ and 711.1–2925.4 U L⁻¹, with detection limit of 17.7 U L⁻¹. The proposed fluorescent assay was applied to TYR activity measurement in human serum samples. It showed promising potential for TYR activity assay in clinical applications.

Abbreviations: TYR, Tyrosinase; CQDs-Dopa, Dopamine functionalized carbon quantum dots; Dopa, Dopamine; PET, Photo-induced electron transfer; CQDs, Carbon quantum dots; QDs, Quantum dots; HR-TEM, High resolution transmission electron microscopy; FT-IR, Fourier transform infrared spectra; MWCO, Molecular weight cut-off; AcH, Acetylcholine esterase; GDH, Glucose oxidase; PPL, Porcine pancreatic lipase; PBS, Phosphate buffered saline; QE, Quenching efficiency.

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1. Introduction

Tyrosinase (TYR, EC 1.14.18.1) is a copper-containing polyphenol oxidase widely existed in microorganisms, plants and animals [1]; it plays pivotal roles in biosynthesis of melanin [2,3], which is involved in color determination of mammalian skin and hair. Abnormal elevated level of TYR can cause excessive production of melanin pigmentation that is one of the serious esthetic problems in human beings [4]. Inhibitors of TYR have been explored to develop whitening products in cosmetic industry. The TYR activity is considered as one of the important biomarkers for melanoma cancer because the enzyme is usually over-expressed in melanoma cancer cells [5]. Furthermore, as the rate-limiting enzyme for the production of neuromelanin, TYR has been reported to be associated with Parkinson disease [6,7]. Therefore, development of highly sensitive assay for TYR activity is of great importance for both basic research and practical application in clinical diagnosis.

Various methods based on colorimetric, electrochemical, and fluorescent assays have been developed to measure TYR activity. Among them, traditional colorimetric assays can directly and quantitatively measure the TYR activity based on the characteristic absorbance of dopaquinone intermediates [8]. However, the accuracy and sensitivity are hindered by the instability of intermediate products. Fluorescent assays based on inorganic semiconductor quantum dots or noble metal nanoclusters generally possess higher sensitivity [9]. But there are concerns about the toxicity for inorganic semiconductor quantum dots (QDs) [10,11], and the inconvenience of using stabilizing agents for noble metal nanoclusters in aqueous solution [9,12].

In recent years, carbon quantum dots (CQDs) have attracted considerable interests due to the good stability, easiness to prepare [13–16] and low biological toxicity [17–19], therefore they have been widely applied in biological analytical chemistry to detect biomolecules such as DNA, micro RNA, trypsin, thrombin, hyaluronidase, and alkaline phosphatase [20–23]. Up till now, there is few report on the application of CQDs towards in the measurement of TYR activity except for Chai et al. [24]. The CQDs was prepared with concentrated acid treatment method, which is not environmentally friendly and dangerous to carry out. In addition to concentrated acid treatment, there have been various synthetic methods for preparation of CQDs including ultrasonic treatment of active carbon [15,23,25], UV irradiation of organic precursors [26], electrochemical oxidation of carbon fiber and thermal treatment of organic precursors [27]. Among all those methods, thermal treatment of organic precursors is appealing due to their simplicity, versatility, and low cost of raw materials [28–30]. Hydrothermal method is the most commonly used one for its environmental friendliness, while CQDs prepared in this way possess advantages of uniform particles, good water solubility and dispersibility [30]. Therefore, CQDs prepared in this way possess great potential for TYR analysis.

In this study, we prepared dopamine functionalized carbon quantum dots (CQDs-Dopa) by one step hydrothermal method for the first time to develop a simple and sensitive fluorescent assay for TYR. UV–Vis absorption, fluorescence spectroscopy, high resolution transmission electron microscopy (HR-TEM) and flourier transform infrared spectroscopy (FT-IR) were used to characterize the prepared CQDs-Dopa. When mixed with solutions containing TYR, the dopamine moiety in CQDs-Dopa conjugate was catalyzed to dopaquinone, which could introduce an intra-particle photo-induced electron transfer (PET) process with CQDs to quench the fluorescence of the original CQDs-Dopa. Quantitative assessment of TYR activity was established based on the fluorescence signal readout. Furthermore, we studied the specificity of this method by comparing the response of some other proteins to CQDs-Dopa, including acetylcholinesterase (AchE), glucose oxidase (GOX), porcine pancreatic lipase (PPL), and human serum albumin (HSA). Influence of the biological metal ions on fluorescence quenching of the CQDs-Dopa by TYR was also investigated.

2. Experimental

2.1. Materials and reagents

Dopamine hydrochloride was purchased from J&K scientific Ltd (Beijing, China). Mushroom TYR (330 U mg⁻¹) was purchased from Solarbio Technology Co., Ltd (Beijing, China). Quinine sulfate was purchased from Aladdin Company (Shanghai, China). Glucose oxidase (GOX) was purchased from Beijing Zhijie Square Technology Co., Ltd. (Beijing, China), levodopa, acetylcholine esterase (AchE), porcine pancreatic lipase (PPL) and human serum were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Human serum albumin (HSA) was purchased from Shuangliu Zhenglong biochemical products research center (Sichuan, China). Citric acid, potassium dihydrogen phosphate (KH₂PO₄), dipotassium hydrogen phosphate trihydrate (K₂HPO₄ 3H₂O), calcium chloride (CaCl₂), sodium chloride (NaCl), potassium chloride (KCl), and barium chloride (BaCl₂) were purchased from Guangdong Guanghua Sci-Tech. Co., Ltd (Guangdong, China). Magnesium chloride (MgCl₂), ferric chloride (FeCl₃ 6H₂O), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Tianjin Kemiu Chemical Reagent Co., Ltd. (Tianjin, China), HPLC grade water was produced by a Milli-Q (18.2 MΩ) system (Millipore, Bedford, MA, USA). The details about preparation of solutions were described in the supplementary information.

2.2. Apparatus

The Ultraviolet spectrums were recorded on an UV-1800 spectrophotometer (Shimadzu, Japan). Fourier transform infrared spectra (FT-IR) were recorded in KBr by a Perkin-Elmer FT-IR spectroscopy. The pH values were measured with a pHS-3 digital pH meter (Shanghai LeiCi Device Works, China). Fluorescence spectra were recorded on an RF-5301 fluorescence spectrometer equipped with a 150-W xenon lamp and 1.0-cm quartz cell (Shimadzu, Japan). High resolution transmission electron microscopy (HR-TEM) images obtained by JEM2100 (JEOL Co., Japan). Enzymatic reactions were incubated in a ZHWY-200H constant temperature shaking table (Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd, China). Mixture was centrifuged by a D3024R centrifuge (SCILOGEX, America), and then freeze-dried by a MODULYOD-230 vacuum freeze drier (Thermo, America).

2.3. Preparation of CQDs-Dopa

The CQDs were prepared according to hydrothermal method with minor modification [31,32]. Briefly, 2.1 g of citric acid and 1.9 g of dopamine hydrochloride were dissolved in 20 mL of deionized water to be heated at 200 °C for 5 h. After reaction, the mixture was cooled to room temperature, and the supernatant was then transferred to a 1000 molecular weight cut-off (MWCO) of dialysis bag to be dialyzed in the deionized water for 24 h for removal of the unreacted species, and then the mixture was centrifuged at 13700 g at 4 °C for 2 min to remove the quantum dots which have been oxidized. Finally, the purified CQDs were collected and freeze-dried before further characterization and application.

2.4. Optimization for enzyme activity detection conditions

Phosphate buffer has a strong buffering capacity in a broad
range of pHs since it has a triple pKₐ, so we chose it in our experiment. To obtain high sensitivity for TYR detection, pH value of phosphate buffered saline (PBS) and incubation time were investigated. To optimize the pH value of the buffer, the fluorescence spectra of CQDs-Dopa were recorded in the absence and presence of TYR under different pH value of 2.0, 4.0, 6.0, 6.2, 6.4, and 6.5, respectively. The incubation time was then optimized under the optimal buffer pH value. The fluorescence spectra of CQDs-Dopa were recorded in the presence of TYR after incubation time of 0, 5, 10, 15, 30, 45, 60, and 90 min, respectively. The fluorescence spectra of the mixtures containing 31.0 µg CQDs-Dopa and different amounts of TYR were monitored by fluorescence spectrometer. The activity of TYR used in optimization, selectivity and interference experiments were 14444 U L⁻¹.

2.5. Specificity and interference of the TYR assay

To investigate the specificity of the developed fluorescent assay, interactions between TYR, AchE, GOX, PPL and HSA to the CQD-Dopa were compared. TYR, AchE, GOX, PPL and HSA were incubated with CQDs-Dopa, respectively, and then monitored by fluorescence spectrometer. All these experiments were repeated for three times.

As a metal-containing enzyme, TYR can possibly be influenced by metals [33]. Therefore, further interference experiments were performed to investigate the effects of several kinds of biologically significant metal ions (Ca²⁺, Ba²⁺, K⁺, Mg²⁺, Na⁺, and Fe³⁺) on the activity of TYR. The concentrations for all the metal ions were 0.1 M. The fluorescence spectra of the mixtures containing metal ions, 31.0 µg CQDs-Dopa and TYR were monitored by fluorescence spectrometer.

2.6. Measurement of TYR activity in human sera

TYR activity was determined according to the increase of absorbance at 475 nm accompanying the consumption of the substrate Levodopa. One unit of the enzymatic activity was defined as the amount of TYR which causes increase of 0.001 in A475 per minute at pH 7.2 at 30 °C, in a 3 mL reaction mix containing Levodopa. Under the optimized pH value and mixing time, the CQDs-Dopa was incubated with human serum samples spiked with various amounts of TYR (1.4, 1.5, 2.9, 3.8, and 4.1 µg), and the fluorescence of the supernatants was recorded. Quenching of CQDs-Dopa’s fluorescence is proportional to TYR activity, thus TYR activities could be obtained by directly readout from the calibration curve. In the meantime, since colorimetric method has been widely used to measure the TYR activity, we performed colorimetric measurement of the TYR in this work to compare the performance of our method [34]. Briefly, 5.0 mM of levodopa was prepared in 0.01 M Tris-HCl buffer and placed in a series of 5.0 mL centrifuge tubes, 2.8 mL of which were incubated at 30 °C for 5 min, and then 0.2 mL of TYR of various concentrations were quickly added into the solution. Absorbance of the mixture at 475 nm was measured once a minute. TYR activity was obtained by calculating the slope of the time line through measuring the optical density at 475 nm.

3. Results and discussion

3.1. Characterization of CQDs-Dopa

UV–visible absorption and fluorescence spectra of CQDs-Dopa measured with a UV–visible and a fluorescence spectrometer were shown in Fig. 1A and B. The CQDs-Dopa showed the maximum absorption at 280 nm and 310 nm. The CQDs-Dopa showed the maximum UV absorptions at 280 nm and 310 nm. The absorption at 280 nm is probably ascribable to dopamine moiety in CQDs-Dopa, which can be concluded from the comparison of dopamine, CQDs-Dopa and citric acid as shown in Fig. 1A. We examined the fluorescence behaviors of CQDs-Dopa under 280 and 310 nm, and found that CQDs-dopa exhibited fluorescence only under the excitation wavelength at 310 nm (Fig. 1B). Therefore, excitation wavelength was set at 310 nm, and a fluorescence emission at 499 nm under this exciting wavelength at 310 nm. The morphology of CQDs-Dopa was characterized by HR-TEM and it was found that they were spherical particles with diameter ranging from 1.0 to 3.0 nm (2.7 ± 0.5 nm) as seen in Fig. 1C. Additionally, freeze-dried CQDs-Dopa was dissolved into PBS buffer (0.01 M, pH 6.0) to prepare 10.1 µg ml⁻¹ CQDs-Dopa solution after storage of 1, 2, 5, 15, 30 and 60 days at 4 °C, and then analyzed by fluorescence spectrometer. CQDs-Dopa was found stable even after 60 days of stock as reflected by the little change in the fluorescence intensity (Fig. 1D).

The formation of CQDs-Dopa was characterized by FT-IR as shown in Fig. 2. The broad absorption at 3195 cm⁻¹ was ascribed to O-H stretching absorption. Absorption at 812 cm⁻¹, 871 cm⁻¹ and 954 cm⁻¹ were ascribed to bending vibration of benzene ring. The absorption at 1120 cm⁻¹ was ascribed to the C-N vibration. The absorption at 1646 cm⁻¹, 1568 cm⁻¹ and 1403 cm⁻¹ were ascribed to C-O stretching vibration, N-H deformation and C-N stretching, respectively, all of which were arising from amide, suggesting that the CQDs-Dopa was synthesized via amide bond [31].

3.2. Optimization of detection conditions

To optimize the detection condition, a series of experiments regarding the pH of buffer and reaction time were carried out. Enzymes’ activities are mostly influenced by pH values, so we first investigate the effects of different pH values on the TYR activity. TYR and CQDs-Dopa were mixed in 0.01 M of phosphate buffer at various pH values of 2.0, 4.0, 6.0, 6.2, 6.4, and 6.5, respectively, to have the enzymatic reaction lasting for 4 h. The fluorescence quenching efficiency (QE) was calculated using the following equation:

\[
QE \% = \frac{F_0 - F}{F_0} \times 100\%
\]

F₀ and F stand for the fluorescence intensity of CQDs-Dopa in the absence and presence of quenchers, respectively. The value of QE reached the highest when pH was 6.0, suggesting that phosphate buffer at pH 6.0 is the mostly appropriate one for TYR activity measurement in this method (Fig. 3A).

Different enzymatic reaction usually needs different period of time. To obtain the best incubation period in this method, the fluorescence of the enzymatic mixture of CQDs-Dopa and TYR were recorded at different time points of 0, 5, 10, 15, 30, 45, 60, and 90 min, respectively. The fluorescence quenching degree increased abruptly within 20 min, while reaching to the platform after 30 min as shown in Fig. 3B, suggesting that the enzymatic reaction completed in 30 min. Therefore, the phosphate buffer of pH 6.0 and incubation time of 30 min were decided to be the optimal condition for the enzymatic reaction.

3.3. Detection assays for TYR activity

The fluorescence intensity of CQDs-Dopa varied with the amounts of TYR added to the solution, therefore, TYR activity can be quantitatively analyzed with the proposed method. It is worth noting that the process of this enzymatic reaction is easily visualized, for the color of the reaction solution quickly changed from light yellow to brown with the addition of TYR. As shown in Fig. 4, the fluorescence intensity at 499 nm continuously decreased with
the increase of the amount of TYR added to CQDs-Dopa solution. Since all the assays have the same reaction time, we described the reaction rate in y-axis by the fluorescence quenching degree \((F_0 - F)\). TYR activities used in the experiments have already been known and described in x-axis. Linear relationship of the fluorescence quenching degree versus TYR activities can be fitted to obtain the calibration. In particular, the fluorescent intensity decrease \((F_0 - F)\) versus various amounts of TYR displayed two linear ranges, i.e. 44.4–711.1 U L\(^{-1}\) and 711.1–2925.4 U L\(^{-1}\). The regression equations were \((F_0 - F) = 0.861 + 0.079 \text{ [TYR]} (R^2 = 0.996)\) and \((F_0 - F) = 27.33 + 0.042 \text{ [TYR]} (R^2 = 0.999)\), respectively. In that similar previous report, carbon nanoparticles (CNPs) were prepared by hydrothermal method at 180 °C for 6 h [32], while we just modified that with a little more higher temperature at 200 °C and shorter processing time of 5 h. As a matter of fact, hydrothermal method has been the easiest and most efficient method as far as preparation of carbon dots is concerned. Meanwhile, the limit of detection (LOD) of TYR was determined as three times the standard deviation of the mean blank signal based on the calibration curve of \(y = 0.079 x + 0.861\), which was calculated to be 17.7 U L\(^{-1}\). Meanwhile, when using colorimetric method to analyze TYR, the linear range was found to be from 297.3 to 16324.3 U L\(^{-1}\), and the LOD was 126.8 U L\(^{-1}\). The whole analytical time for TYR activity was around twenty minutes, which is approximately the same with our method (Supplementary Material). Although colorimetric method

Fig. 1. (A) UV–Visible absorption spectra of CQDs-Dopa, Dopamine and Citric acid. (B) Fluorescence spectrum of CQDs-Dopa from 290 to 600 nm excited at 280 nm and 310 nm light. (C) HR-TEM image of CQDs-Dopa. (D) Stability of CQDs-Dopa (10 \(\mu\)g ml\(^{-1}\)) after storage of 1, 2, 5, 15, 30 and 60 days at 4 °C.

Fig. 2. FT-IR spectra of CQDs-Dopa.
possesses a broader linear range, it is much less sensitive than our method. Furthermore, a comparison of our method with several previously reported TYR detection assays based on fluorescent detection is shown in Table 1.

Preparation of the sensors involved in those assays most likely requires complex pre-treatments, tedious procedures, too much time and/or dangerous reagents. Hydrothermal method provides more facile means to overcome these drawbacks. To the best of our knowledge, this is the first application of CQDs-Dopa synthesized with hydrothermal method to TYR analysis. It is noted that a similar CQDs was prepared with the same method to detect dopamine and iron ion [32], in which dopamine was used as both carbon source and CQDs surface modifier. However, our synthetic strategy is different in that citric acid was used as the carbon source to produce CQDs, and dopamine was bond to the CQDs’ surfaces while they formed. Because of the difference in synthesis strategy, reaction conditions used for the synthesis were also modified to achieve an optimal result.

Based on the proposed CQDs-Dopa, our analytical method offers advantages of shorter analytical time, good sensitivity and broad ranges. The proposed fluorescence assay for TYR provides a good performance.

### 3.4. Selectivity and interference studies

Selectivity is a very important indicator to evaluate the performance of the analytical method. Since our fluorescent assay is for the TYR present in the serum, it is necessary to evaluate the influence of other proteins on this CQD-Dopa based method. The following proteins were incubated with CQD-Dopa respectively, namely AchE, GOX, PPL and HSA, and the results were compared with that of TYR as shown in Fig. 5A. There was almost no QE observed for those proteins, suggesting that CQDs-Dopa possess high specificity against TYR.

The activity of TYR is possibly affected by metals for it is a metal containing enzyme. The coexisting metal ions interference experiments were performed in the presence of TYR. As shown in Fig. 5B, all those metal ions except for iron ions exhibited little effects on the QE of the CQDs-Dopa by TYR. When iron ions were added into the reaction system, the quenching degree is enhanced, which
fluorescence method can quantify TYR in two wide linear ranges, i.e., 44.4 to 711.1 and 711.1–2925.4 U L⁻¹ with a low detection limit of 17.7 U L⁻¹. The performance of this fluorescent assay matches that obtained from the colorimetric method in human serum samples, indicating that it could be ascribed to the quenching effect of iron ions [32].

3.5. Detection of TYR in human serum samples

The proposed fluorescent assay was applied for the measurement of TYR activity in five human serum samples. At the same time, the human serum samples were analyzed by the colorimetric method for comparing the performance of our method. The TYR activities in all human serum samples were calculated from the standard curves and regression equations. The analytical results are listed in Table 2, together with the t-values calculated from comparing these results by t-test. When confidence is set at 95% and degree of freedom equals 4 (i.e., 3 + 3/2), the tabulated value of t equals 2.776. As shown in Table 2, the values of t calculated are all less than 2.776, which indicates that the proposed fluorescent method is statistically comparable with the widely accepted colorimetric method in terms of assay accuracy. The results from both methods were in agreement with each other in two linear ranges, which suggested that the proposed fluorescent assay possess accuracy in analyzing TYR activity. Therefore, this proposed fluorescent assay has potential for broadening avenues of detecting TYR activity in actual human serum samples.

4. Conclusions

In conclusion, we have developed a simple, practical and sensitive fluorescent method for TYR detection. The CQDs-Dopa was synthesized through a simple one-pot hydrothermal method without any stabilizing agents, which formed through amide bond between citric acid and dopamine. The dopamine moiety in CQDs-Dopa conjugate was catalyzed into dopaquinone, and an intra-particle PET process consequently occurs between the latter and CQDs. As a result, the fluorescence for the original CQDs-Dopa was quenched, and it is easily visualized that the color of the reaction solution changed from light yellow to brown after addition of TYR into the CQDs-Dopa solution. The proposed fluorescent assay can quantify TYR in two wide linear ranges, i.e., 44.4 to 711.1 and 711.1–2925.4 U L⁻¹ with a low detection limit of 17.7 U L⁻¹. The performance of this fluorescent assay matches that obtained from the colorimetric method in human serum samples, indicating that

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Table 1

| Analytical materials | Analytical time (min) | Detection limit (U L⁻¹) | Linear range (U L⁻¹) | References |
|----------------------|-----------------------|------------------------|----------------------|------------|
| Dopa-CQDs            | 20                    | 7.0                    | 232.0–793.5          | [24]       |
| AuNCs@TYr            | 240                   | 80.0                   | 500.0–2000.00        | [33]       |
| AuNCs                | 90                    | 6.0                    | 6.0–3600.0           | [35]       |
| Dopa-Au/Ag NCs       | 10                    | 13.5                   | 45.0–319.5           | [36]       |
| CQDs-Dopa            | 30                    | 17.7                   | 44.4–711.1           | This work  |

Table 2

Comparison of detection of TYR in human serum samples by the colorimetric method and the proposed fluorescent assay.

| Samples            | Colorimetric method (U L⁻¹) | Confidence Intervals (95%) (U L⁻¹) | Fluorescence method (U L⁻¹) | Confidence Intervals (95%) (U L⁻¹) | t-value calculated |
|--------------------|----------------------------|-----------------------------------|-----------------------------|-----------------------------------|--------------------|
| Serum 1            | 400.0 ± 10.3               | 361.6–438.4                       | 389.0 ± 25.7                | 350.6–427.4                       | 0.56               |
| Serum 2            | 444.4 ± 11.5               | 411.3–478.0                       | 458.2 ± 21.2                | 424.9–491.5                       | 0.81               |
| Serum 3            | 865.1 ± 22.3               | 838.2–892.0                       | 854.3 ± 17.4                | 827.4–881.3                       | 0.39               |
| Serum 4            | 1111.1 ± 28.7              | 1030.3–1191.9                     | 1157.2 ± 37.1              | 1076.4–1237.9                     | 1.21               |
| Serum 5            | 1222.2 ± 31.6              | 1170.2–1274.2                     | 1265.8 ± 32.7              | 1213.9–1317.9                     | 1.14               |

At a confidence level of 95% and degree of freedom of 4 (i.e., 3 + 3/2), the tabulated value of t equals 2.776. This work
this method is practical and can be reliable. This fluorescent assay possesses promising potential in TYR activity measurement in actual samples for its good linear relationship and sensitivity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2017.09.038.

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