A STUDY ON THE USE OF CARBON QUANTUM DOTS ON hCG IMMUNE ANALYSIS

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ABSTRACT

Quantum dot – antibody conjugations are of potential materials for diverse bioanalysis, diagnosis and medical treatment applications. Herein, we present the synthesis of human chorionic gonadotropin (hCG) – carbon quantum dot (CQD) conjugate and its application in immune analysis of hCG antigen. By comparing with the standard analysis procedure, it has been revealed that hCG-CQD conjugation can be used for the analysis of hCG antigen with a detection limit of about ng/ml.

Keywords: *Carbon quantum dots; human chorionic gonadotropin; antigen; immunoassay; photoluminescence.*

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NGHIÊN CỨU SỬ DỤNG CHẤM LƯỢNG TỬ CARBON TRONG PHÂN TÍCH hCG

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TÓM TẮT

Gắn chấm lượng tử (QDs) vào kháng thể để tạo thành vật liệu liên hợp kết hợp được tính đặc hiệu của kháng thể và tính chất huỳnh quang của QDs có tiềm năng ứng dụng lớn trong phân tích sinh hóa, chuẩn đoán và điều trị. Trong bài báo này, chúng tôi trình bày kết quả nghiên cứu gắn chấm lượng tử carbon (CQD) vào kháng thể human chorionic gonadotropin (hCG) và đánh giá khả năng ứng dụng của vật liệu liên hợp thu được (hCG-CQD) trong phân tích kháng nguyên hCG bằng phương pháp miễn dịch huỳnh quang. So sánh kết quả phân tích trên 20 mẫu nghiên cứu với kit chuẩn cho thấy hCG-CQD có thể được sử dụng để phân tích hCG với giới hạn phát hiện cỡ ng/ml.

Từ khóa: *chấm lượng tử carbon; human chorionic gonadotropin; kháng nguyên; miễn dịch; huỳnh quang.*

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1. Introduction

hCG is a hormone comprised of α-(93-amino acid, 14.5 kD) and β-(145-amino acid, 22.2 kD) subunits. While the α-subunit is common to all members of the glycoprotein hormone family the β-subunit is unique to hCG owing to its C-terminal peptide [1]. hCG is produced by trophoblast cells during early pregnancy and represents key embryonic signals essential for the maintenance of pregnancy. The concentration of β-hCG increases rapidly after implantation; its levels in serum and urine reach maximum values after 8 to 10 weeks and then decrease gradually [2]. Therefore, analysis of β-hCG levels in a wide range of variety provide important information for diverse clinical situations, such as diagnosis and monitoring of pregnancy and pregnancy-related disorders, prenatal screening, Down syndrome and gynecological cancers [3]–[6].

Immunofluorescence has been used widely for the analysis of hCG because of many advantages, such as short acquiring time, large range of concentrations and the fact that the fluorescence signal is not affected by background emission [7], [8]. In this method, a half of couple hCG is immobilized on a solid plate while the other half of the couple is labelled with fluorescent agent. In our previous study, we used Eu^{3+} labelled hCG for the immunofluorescence analysis of hCG that exhibited a LOD (limit of detection) of 11.9 ng/ml and a LOQ (limit of quantification) of 17.9 ng/ml [8]. The fundamental drawback of using hCG labelled with Eu^{3+} complexes is the narrow photoluminescence excitation range of the complexes. As for example, the excitation range of Eu-NTA (2-naphthoyltriluoroacetone) is 340 ±10 nm. Additionally, the expensiveness of lanthanide metals would raise the cost for hCG measurements. Recently, quantum dots (QDs) [9] and graphene oxide [10] have been studied to replace the lanthanide complexes in immunofluorescence assays.

Herein, we report the use of amine terminated CQDs as fluorescent agent to synthesize hCG-CQD conjugation and its application in immunofluorescence analysis of hCG.

2. Experimental

2.1. Materials

Polystyrene (PS) plates, PBS (phosphate buffer saline), sodium azide (NaN_3), BSA (Bovine Serum Albumin), (sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate) (SMCC), hCG antibody and hCG antigen were purchased from Thermo fisher. Other chemicals including citric acid pentahydrate 99% (CA), 2-iminothiolane 99% (IMTA), ethylenediamine 99,5% (EDA) and solvents, such as acetone, dimethylsulfoxide (DMSO), phosphate buffered saline (PBS-1X) were purchased from Alladin Chemicals.

2.2. The synthesis of NH_2 – terminated carbon quantum dots

A 250 ml, three-neck flask containing 50 ml of CA solution in glycerol was equipped with sand bath heater, a magnetic stirrer and a Schlenk line system. Under N_2 atmosphere, the solution was heated up 227^{o}C and 10 ml solution of EDA in glycerol was rapidly injected. The amount of EDA was calculated so that the molar -COOH/-NH_2 ratio was 1/2.3. Temperature of the mixture dropped to about 220^{o}C and it was maintained for 30 minutes. The reaction mixture was cooled by water. To purify CQDs, acetone was added to the reaction mixture to precipitate CQDs which were then collected by mean of centrifugation at 8000 rpm for 10 minutes at 5^{o}C. Solid CQDs were dispersed in deionized (DI) water and precipitated again with acetone. This process was repeated three times to remove completely glycerol as well as unreactive precursors. Next, solution of CQDs in DI water was filtered through

0.21µm PTFE membrane filters to remove large CQD aggregates. Finally, CQDs solution was dialyzed with a pore size cutoff of 2000 Dalton against DI water for 24 hours to remove small particles.

2.3. The synthesis of hCG-CQD conjugation

The stepwise synthesis of hCG-CQD conjugation is schematically illustrated in Fig. 1.

2.3.1. The synthesis of CQDs having SMCC binder

After adding 2.2 µl solution of SMCC in DMSO (10 mg/ml) to 1 ml solution of CQDs in DMSO (100 mg/ml) the mixture was vortex mixed for 30 minutes. Unreacted SMCC was washed out by precipitation with ethanol. Finally, CQD-SMCC was dissolved in PBS-1X buffer with a concentration of 4.3 mg/ml.

2.3.2. Functionalization of β-hCG with SH groups

Add sequentially 42 µl solution of IMTA (10 mg/ml) and 40 µl PBS-1X into a tube containing 8 µl hCG solution (4750 µg/ml) and mix the mixture for 15 minutes. hCG-SH was purified by mean of column chromatography using silica as stationary phase and PBS-1X as the eluent. The concentration of hCG-SH was determined by calibrating to the absorbance of solution at 280 nm to be 400 µg/mL.

2.3.3. Binding hCG-SH and CQD-SMCC

Mix 1 ml of CQD-SMCC and 1 ml of hCG-SH solution for 30 minutes prior to adding 6 µl of aqueous solution of NaN_3 (5%) and then the mixture was stored in dark at 4°C until use.

2.4. hCG analytic process

2.4.1. Building up the standard curve

Standard solutions of hCG antigen with concentrations of 10.6, 106, 1030, 5180 and 10100 ng/ml were prepared from the original solution and PBS 0,01M. Add sequentially 150µl of PBS-1X and 25µl of the standard hCG antigen solution into polystyrene plates which were previously coated with hCG antibody [8]. Next, 15µl of hCG-CQD solution was added and the mixture was cultured for 2 hours prior to washing three times with PBS-1X to remove unreacted hCG-CQD. Finally, 50µl of PBS-1X was added and fluorescence intensity at 480 nm was recorded under excitation at 360 nm. The standard curve was obtained by fitting the dependence between hCG concentration (y) and fluorescence intensity (x) using OriginPro 8RS.

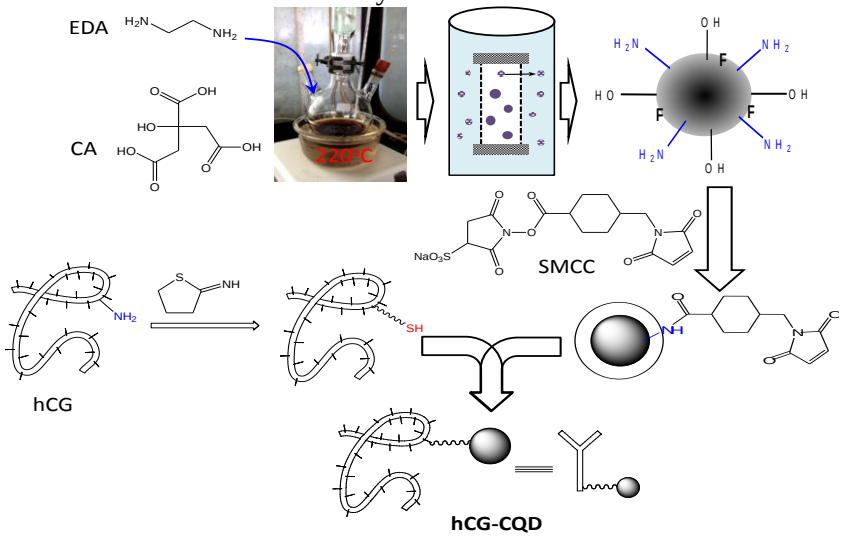

Figure 1. *Procedure to prepare hCG-CQD conjugation*

2.4.2. Analysis of hCG samples

20 hCG samples were randomly selected, marked and divided into two parts. One was analyzed using the procedure described in 2.4.1 the other part was analyzed using a standard kit (DELFIA® hCG kit, Perkin Elmer). The analysis procedure is illustrated in Fig. 2.

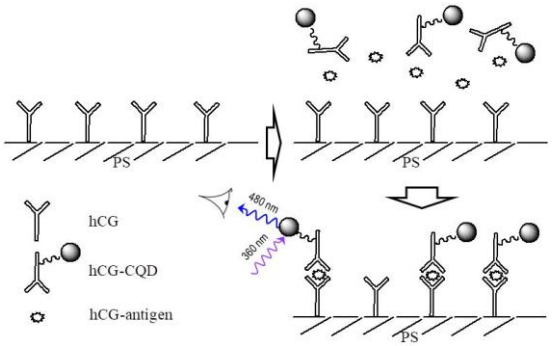

Figure 2. *Procedure for the analysis of hCG using hCG-CQD conjugation.*

2.5. Characterizations

UV-Vis absorption spectra of CQDs aqueous solution was conducted on a UV-2450 (SHIMADZU). Photoluminescence (PL) and photoluminescence excitation (PLE) spectra of CQDs solutions were measured on a Nanolog® (HORIBA Scientific). Infrared (FTIR) spectra of solid CQDs were carried out on JASCO FT/IR6300. X-ray photoelectron (XPS) spectra of CQDs was performed on a PHI 5000 VersaProbe II. Transmission electron microscopy (TEM) images of CQDs were obtained on a JEM 2100 (JEOL).

3. Results and discussion

3.1. The structure of carbon quantum dots

Characterization results of CQDs are summarized in Fig. 3. TEM image shown in Fig. 3a exhibits CQDs as dark spheres, which have a diameter varying from 4.5 to 10 nm. We rarely observed lattice fringes on CQDs, indicating that CQDs were mostly amorphous. Additionally, CQDs had different degree of carbonization because their darkness in the TEM image varied. These observations were similar to those of CQDs synthesized from CA and EDA by a hydrothermal method [11]. Chemical analysis by XPS method shown in Fig. 3b improves that CQDs were composed of C, N and O elements. High-resolution XPS spectrum for C 1s shown in Fig. 3b' confirmed that C presented in CQDs in the forms of C-C, C-N and C-O or C=O whose binding energies are 284.6 eV, 285.7 eV and 287.4 eV, respectively. Additionally, XPS spectrum of N 1s shown in Fig. 3b'' confirms that N were mainly in pyridinic (398.4 eV), pyrrolic (399.5 eV) and graphitic (401.1 eV) structural types. Vibration peaks of important groups were observed in the FTIR spectrum and noted in Fig. 3c including –N-H (3400 cm^{-1}), =C-H (3100 cm^{-1}), -C-H (2800 – 3000 cm^{-1}), NC=O (1650 cm^{-1}), O=CNH (1570 cm^{-1}). The existence of amide (O=C-NH) and amine (N-H) groups in the absence of acidic carbonyl (O=C-OH) groups strongly suggests that CQDs were decorated with amine (-NH_2) groups on the surfaces together with well-known surface fluorophores (derivative of citrazinic acid) [11]–[13]. Based on these characterizations, we modeled CQDs as shown in Fig. 3d. CQDs involved a carbogenic core that included polyaromatic structures embedded in a hydrocarbon matrix; surface fluorophore as shown in red and surface polar groups shown in blue.

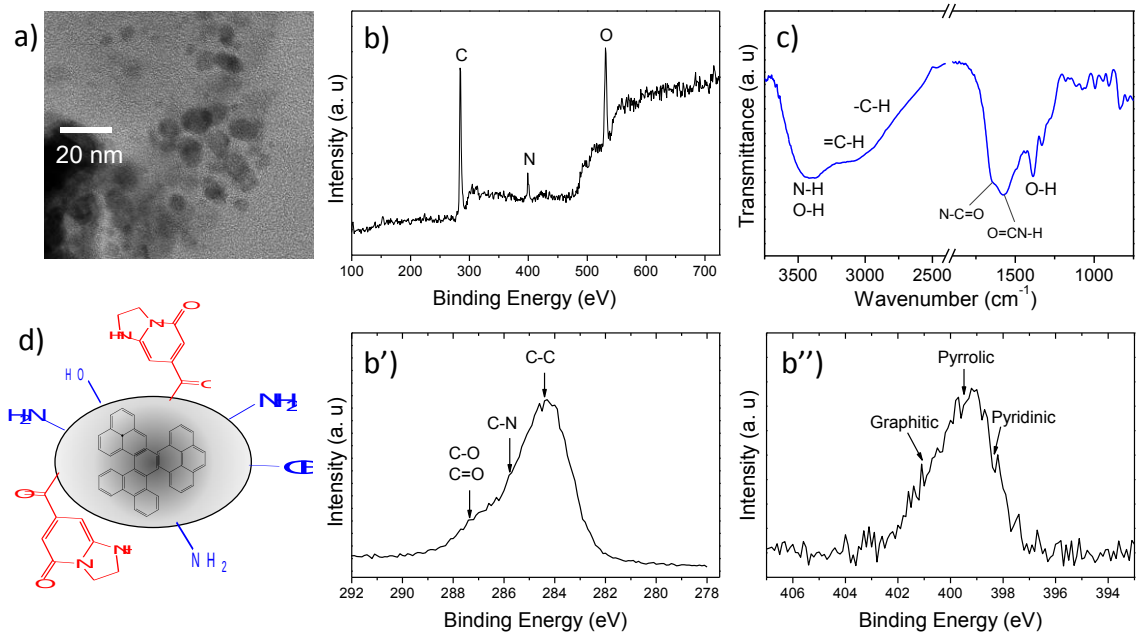

Figure 3. *a) TEM, b) XPS survey spectrum, c) FTIR spectrum and d) model structure of CQDs. b') and b'') are high-resolution XPS spectra of C 1s and N 1s, respectively.*

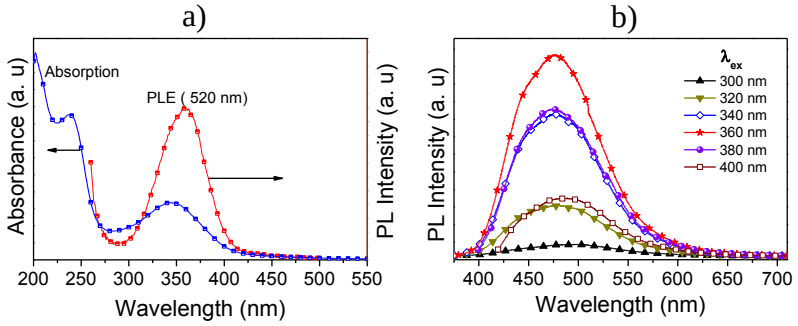

Figure 4. *a) The UV-Vis absorption and PLE (observed at 520 nm), and b) PL spectra of CQDs.*

3.2. The optical properties of CQDs and hCG-CQD conjugations

The UV-Vis, PLE and PL spectra of CQDs are summarized in Fig. 4. It is obviously from Fig. 4a that the absorption and the excitation spectra of CQDs showed a common broad peak maximized at about 357±3 nm. This is the characteristic peak of the surface fluorophores [13]. The PL spectra of CQDs were varied with excitation wavelength as seen in Fig. 4b. PL intensity reached maximum values when excited at about 360 nm. Additionally, PL intensity maximized at 480 nm and it was independent to the excitation wavelength. These results suggest that the optical properties of CQDs were dominated by the surface fluorophore [12], [13].

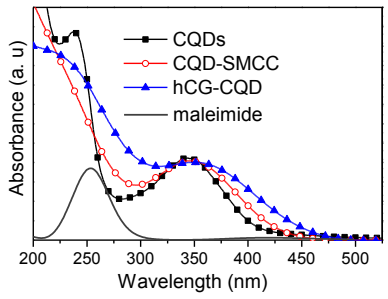

Figure 5. *UV-Vis absorption of CQDs, CQD-SMCC and hCG-CQD normalized at 355 nm.*

Thank to surface amine groups, CQDs were easily decorated with SMCC via the reaction between the amine groups and N-hydroxy succinimide-ester head of SMCC. Due to maleimide group of SMCC has a

characteristic absorption band in 200-300 nm (maximum at 256 nm), the absorption shoulder of CQDs at 245 nm were blurred in CQD-SMCC as well as in hCG-CQD conjugation. Similarly, the absorbance of hCG-CQD conjugation near 280 nm increased as compared with CQDs or CQD-SMCC because hCG absorbs light near 280 nm. Importantly, the characteristic absorption band of the surface fluorophore 355 nm was still visible in the hCG-CQD conjugation. This observation indicates that the conjugation of hCG to CQDs via SMCC link does not alter the surface fluorophore; hence the fluorescent properties of CQDs.

Table 1. *Comparison the analysis results using hCG-CQD and the standard kit.*

STT	β-hCG (ng/ml)		Deviation (%)	STT	β-hCG (ng/ml)		Deviation (%)
	Standard kit	hCG-CQD			Standard kit	hCG-CQD	
1	489	506	3.5	11	2230	2325	4.3
2	823	817	-0.7	12	2316	2486	7.3
3	858	869	1.3	13	2563	2336	-8.9
4	1356	1400	3.2	14	2650	2475	-6.6
5	1390	1305	-6.1	15	2865	2938	2.5
6	1589	1426	-10.3	16	2905	2705	-6.9
7	1678	1590	-5.2	17	3215	3150	-2.0
8	1765	1826	3.5	18	3547	3605	1.6
9	1878	1905	1.4	19	4575	4750	3.8
10	2050	2095	2.2	20	4650	4550	-2.2

3.3. The analysis of hCG antigen using hCG-CQD conjugation

The analytic results conducted on 20 hCG samples using either procedure in 2.4.1 or standard kit are summarized in Table 1. The experimental results deviated by -10.3-7.3% as compared with the standard procedure. The average deviation was about 4.2%. Additionally, based on the fluorescence intensity on blank samples and the standard curve, LOD and LOQ were estimated according to ref [14] to be about 7.1 and 15.8 ng/ml, respectively.

4. Conclusions

CQDs have been synthesized successfully by a hot injection method. CQDs were spherical with a diameter ranging from 4.5 to 10.3 nm and had amine and fluorophore functional groups on the surfaces. The surface amine groups are useful for preparation of hCG-CQD conjugation via SMCC linker while the surface fluorophore accounts for the optical properties of CQDs as well as resultant hCG-CQD conjugations. It has been demonstrated that hCG-CQD conjugations were successfully used as labelled antibody for immunofluorescence assay with good LOD and LOQ values. The results are of important to deploy non-toxic, fluorescent CQD and its antibody conjugation into diverse field of bioanalyses.

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