Preparation and Characterization of Epitope-Based Ratiometric Fluorescent Molecularly Imprinted Polymers

Xicheng Yang[1], Hongjuan Zhang[2], Hongliang Xin[2] , Yankun Gao[2*]
1.Jinling High School, Nanjing 210029, China.
2. Nanjing Medical University, Nanjing 211166, China.

Abstract: In order to solve the problem of difficult detection of neuronal nitric oxide synthase in the screening of neuronal nitric oxide synthase-postsynaptic density95 (nNOS-PSD95) uncoupling agent, this study used 133 amino acids ($nNOS_{1-133}$) at the nitrogen terminal of nNOS as template molecules, carbon dots and quantum dots as ratio fluorescence recognition elements, SiO_2 as matrix for the first time, combined with surface molecular imprinting technology and antigen-determining principle, to prepare ratiometric flurescent molecularly imprinted polymers (RFMIPs). The resulting RFMIPs were characterized by fourier transform infrared spectroscopy, scanning electron microscopy, transmission electron microscopy and thermogravimetric analysis，exhibiting uniform spherical morphology, which unambiguously confirmed the successful formation of the nanosensor. The result indicates that the synthesized sensors have promising potential for the assay of trace peptides in complex matrices.

Keywords: Fluorescent Nanosensor; Molecularly Imprinted Polymers; Preparation; Characterization

1. Introduction

Molecular imprinting simulates the reaction between antigens and antibodies in living organisms, which can memorize the size and shape of template molecules and interact with the coordination groups of template molecules [1]. The technology using small molecule compounds as templates, has been widely applied in environmental monitoring, food testing and drug separation [2-4]. However, the preparation of molecularly imprinted polymers (MIPs) using biological macromolecules such as peptides and proteins as templates is still challenging [5]. The exposure fragment of the target protein (epitope) with small structure is used as the template molecule to identify the target protein according to the cavity generated by the epitope, which can greatly reduce the uncertainty of the imprinting process and has a broad application prospect in protein detection [6,7].

In order to improve the sensitivity of MIPs detection, fluorescent materials have been introduced into molecular imprinting technology as sensitive sensors [8]. Quantum dots (QDs), a traditional nano-fluorescent material, has been widely used in the field of molecular imprinting, especially in the detection of proteins due to its wide excitation spectrum and narrow symmetric emission spectrum [9]. Carbon dots (CDs), a new type of fluorescent nanomaterial which has attracted great attention from biologists, show good stability and low toxicity in aqueous solution. Therefore, studies on the combination of CDs and molecularly imprinted technology in the field of proteins are also emerging [10]. However, if only the intensity of a single emission wavelength is used to quantitatively detect the target protein, the accuracy of the detection result is lower, because the fluorescence intensity of a single wavelength may be disturbed by light scattering, changes in the microenvironment near the probe, differences in the stability of excitation light source, and changes in protein concentration around the probe. In order to minimize these interference factors, ratiometric fluorescence is introduced into molecular

imprinting, and the concentration of the target protein is determined based on the ratio of the two emitted light intensities, which can improve the accuracy of the detection results.

In addition, QDs are often combined with SiO_2 to prepare fluorescent nanocomposites compatible with water systems and organic systems, which can be used in biological and chemical fields. The commonly used method is to directly wrap the QDs in SiO_2, but this will greatly reduce the fluorescence efficiency of the QDs, and at the same time loss the complete protection of QDs. In order to reduce the fluorescence quenching of QDs, the CdTe QDs were first fixed on the surface of SiO_2 sphere and then coated with a layer of silicon to form a nanocomposite fluorescent sphere with electrostatic interaction between positive and negative particles [11]. Hundreds of CdTe QDs particles were assembled on the surface of the same silicon sphere, which can play the role of amplifying the fluorescence signal layer by layer and improve the efficiency of fluorescence detection.

Considering the above, we have successfully constructed a biomimetic fluorescent nanosensor based on molecularly imprinted polymers modified with CDs and QDs which is also capable of rapid, highly sensitive and selective detection of nNOS in biological samples, where CDs were used as transducer elements and MIPs were used as recognition. The schematic illustration of the procedure is given in Fig. 1.

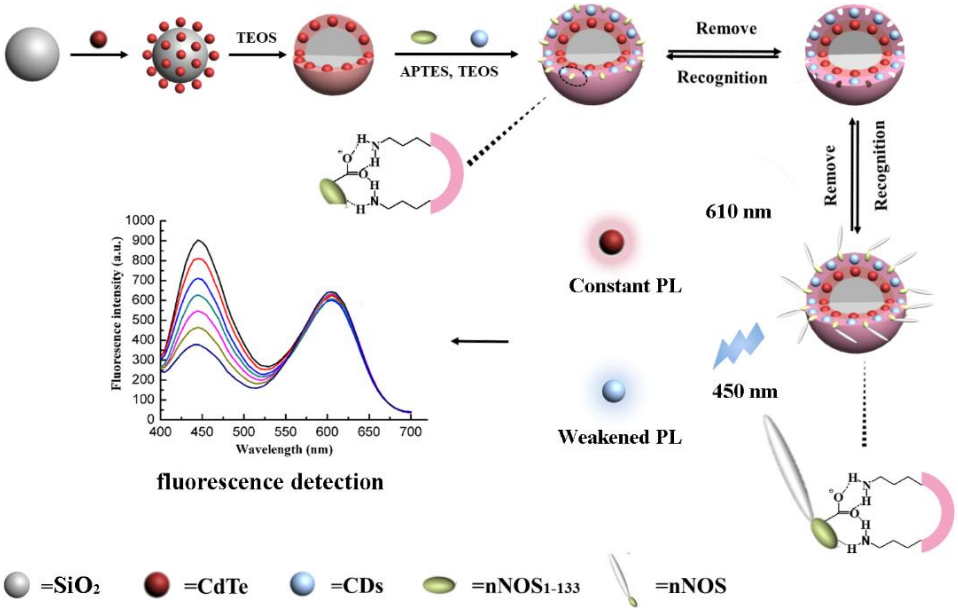

Fig. 1 Scheme of the preparation and the recognition process of RFMIPs

2. Experimental

2.1 Materials and Apparatus

Tellurium power, cadmium acetate ($CdAc_2 \cdot 2H_2O$), 3-mercaptopropionic acid (MPA), 3-aminopropyltriethoxysilane (APTES) and tetraethoxysilane (TEOS) were purchased from Aladdin (Shanghai, China). Sodium borohydride ($NaBH_4$) and ammonia ($NH_3 \cdot H_2O$) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). A shake culture box (ZHLY-180, China) and temperature magnetic mixer of C-MAG HS 7 (IKA Processing Equipment, Germany) were used during the experiment.

2.2 Preparation of SiO_2-NH_2, CdTe QDs and functionalized CDs

Monodisperse amino-functional SiO_2 nanoparticles were prepared according to the Stöber method. 15 mL $NH_3 \cdot H_2O$,

2.5 mL TEOS and 250 mL ethanol were added into a three-necked bottle, stirred magnetically at 40 °C for 12 h. Afterwards, 1.3 mL TEOS/APTES (12:1, v/v) was added dropwise and then the reaction was kept for 12 h. Finally, the product was washed with ethanol and water to neutrality, and then freeze-dried for later use.

The preparation of red fluorescent CdTe QDs is as follows: The reaction system was strictly controlled without oxygen. 1 mM tellurium powder and 10 mM $NaBH_4$ were added into 10 mL water, and continuously stirred until Te was completely reacted, resulting in colorless or pale pink transparent liquid, namely NaHTe solution. Afterwards, 2 mM $CdAc_2 \cdot 2H_2O$ was dissolved in 150 mL ultrapure water, mixed with 4.8 mM MPA, and the pH of the system was adjusted to about 10 with NaOH (1 M) solution. Then the NaHTe solution prepared above was stirred magnetically at 95 °C for 8 h. The product was purified by ethanol and then dried for later use.

Aminosilane-modified CDs were synthesized by a one-step hydrothermal method. 0.8 g sodium citrate was completely dissolved in 16 mL ultrapure water, then 4 mL APTES was added and continuously stirred magnetically for 20 min to mix well. The mixture was then transferred to a 50 mL polytetrafluoroethylene reactor and reacted in an oven at 200 °C for 2 h. Then, the obtained product was dialyzed in a 500 KDa dialysis bag for 24 h and concentrated to 10 mL for later use.

2.3 reparation of SiO_2@CdTe@SiO_2

First, 80 mg SiO_2-NH_2 nanoparticles were added in 20 mL ultrapure water, after ultrasonic dispersion, 1 mg CdTe QDs and 1 mL phosphate buffered saline (PBS, pH 5.5) were added successively. After being magnetically stirred for several minutes, the CdTe QDs were assembled on the surface of SiO_2-NH_2 by electrostatic interaction to generate SiO_2@CdTe. Afterwards, the resulting SiO_2@CdTe were centrifuged, washed with ultrapure water, and dispersed again in 6 mL water. The Stböer method was still used to wrap SiO_2 layer in the outer layer of SiO_2@CdTe. 48 mL anhydrous ethanol and 210 μL APTES were added to the above solution and stirred at 40°C for 3 hours. Then 480 μL $NH_3 \cdot H_2O$ and 240 μL TEOS were added dropwise and reacted for 6 h to form SiO_2@CdTe@SiO_2 (SS). The resulting product SS was centrifuged, washed with anhydrous ethanol to remove impurities, then washed with water to neutrality, and freeze-dried for use.

2.4 Preparation of RFMIPs

2 mg $nNOS_{1-133}$ and 160 μL APTES were added into 20 mL PBS, magnetically stirred and then reacted for 1 h in the dark to form pre-polymerization solution. Afterwards, 10 mL PBS solution was added in which CDs and 20 mg SS were dispersed, and magnetically stirred for 1 h, then 200 μL $NH_3 \cdot H_2O$ and 200 μL TEOS were added, and reacted at 40 °C in the dark for 12 h. The reaction product was eluted with methanol/0.1 M sodium hydroxide (9/1, v/v) to remove $nNOS_{1-133}$, and the remaining methanol and sodium hydroxide were removed with ultrapure water, and then freeze-dried to obtain RFMIPs. The template protein $nNOS_{1-133}$ was not added during the preparation of ratiometric fluorescent molecularly non-imprinted polymers (RFNIPs). The rest of the preparation was the same as RFMIPs.

2.5 Characterization of RFMIPs

The characteristic absorption peaks of the infrared spectrum (FT-IR) in the range of 4000-500 cm^{-1} were investigated and analyzed. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and high-resolution scanning electron microscopy (HESEM) recorded the morphology and structure of the synthesized nanoparticles, and thermogravimetric analysis (TGA) detected the thermal stability of the polymer.

3. Results and discussion

3.1 Preparation of RFMIPs

The preparation of RFMIPs was shown in Fig. 1. In this experiment, SiO_2 with an amino functional group was first prepared. As shown in Table 1, the amino functional group would be protonated under slightly acidic conditions, making SiO_2-NH_2 positively charged. SiO_2-NH_2 and CdTe had a zeta potential of 11 and -33 mv in buffer solution (pH 5.5) respectively. After the formation of SiO_2@CdTe through electrostatic assembly, it was found that the zeta potential changed to -21. The decrease of the zeta potential indicated that CdTe was adsorbed on the surface of SiO_2-NH_2. In order to protect the CdTe on the surface of SiO_2@CdTe, the surface layer of silica was further modified to form SS. After the modification was completed, it was found that the Zeta potential level was equivalent to SiO_2-NH_2.

Table 1 Diameter distribution and Zeta potential of particles

Particles	Diameter/nm	Zeta potential/mv (pH=5.5)
SiO_2	88	-18
SiO_2-NH_2	101	11
CdTe	10	-33
SiO_2@CdTe	122	-21
SS	151	8

3.2 Characterization of RFMIPs

3.2.1 Electron microscope analysis

In this experiment, TEM and SEM were used to analyze the morphology of the nanoparticles synthesized at each step. Fig. 2A and 2B are high-resolution scanning electron microscope images of CDs and CdTe respectively. From the figure, it can be seen that the edges of the two were not clear and the shape was irregular. The particle size of CDs and CdTe was about 11 nm and 10 nm respectively. Fig. 2C-F and Fig. 2c-f were SEM and TEM of SiO_2-NH_2, SiO_2@CdTe, SS, and RFMIPs, respectively. Fig. 2C and Fig. 2c showed that SiO_2-NH_2 was a round shape with smooth surface and the hydrated particle size was about 100 nm. Due to the small particle size of CdTe, the morphology of SiO_2@CdTe was not significantly different from that of SiO_2-NH_2 in the SEM, but it can be clearly seen from the TEM that a large number of black spots were adsorbed on the surface of the silicon sphere, indicating that the connection of CdTe by static electricity was successful. Compared with SiO_2-NH_2, the hydration particle size of SiO_2@CdTe increased by about 22 nm. After further modification of a layer of silicon, the shape of SS was similar to that of SiO_2-NH_2, and it remained a relatively round sphere. No wrapped CdTe was observed from TEM, and the hydrated particle size was about 150 nm. After the preparation of the imprinted layer, the surface of the RFMIPs became rough, the particle size further increased, and the hydrated particle size was about 220 nm. The observation of the electron micrograph showed that each step in the experiment was successfully synthesized.

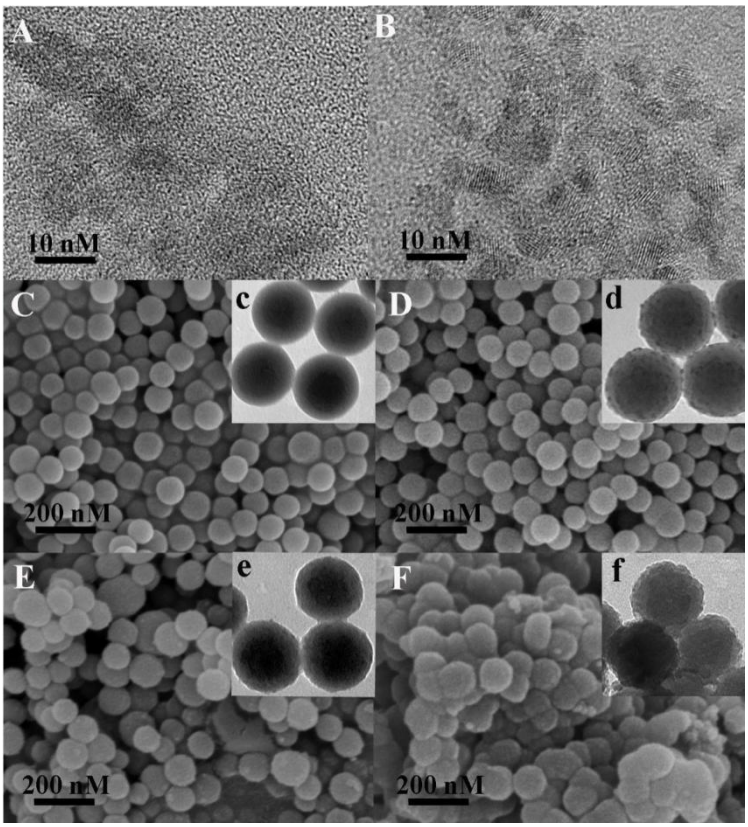

Fig.2 High resolution SEM images of (A) CDs and (B) CdTe; SEM images of (C) SiO_2-NH_2、(D) SiO_2@CdTe、(E) SS and (F) RFMIPs; TEM images of (c) SiO_2-NH_2、(d) SiO_2@CdTe、(e) SS and (f) RFMIPs

3.2.2 Infrared analysis

To verify the successful preparation of RFMIPs, FT-IR scanning was performed on the prepared CDs, CdTe, SiO_2-NH_2, SiO_2@CdTe, SS, and RFMIPs. As shown in Fig. 3A (a), the stretching vibration peaks of the carboxylate on MPA appeared at 1560 cm^{-1} and 1408 cm^{-1}, indicating that the MPA-modified CdTe were successfully synthesized. The absorption at 799 cm^{-1} and 1096 cm^{-1} was caused by the vibration of Si-O-Si. Compared with the infrared curve (3A (b)) of SiO_2-NH_2, the characteristic absorption of MPA was added on the SiO_2@CdTe curve (3A (c)), indicating that CdTe was electrostatically assembled on the surface of SiO_2-NH_2. In 3B (a), the absorption of amino-functionalized CDs at 2800-3000 cm^{-1} and 1403 cm^{-1} was attributed to the stretching vibration and in-plane bending vibration of C-H. The absorption at 1595 cm^{-1} was due to the out-of-plane bending vibration of N-H. SS (3B (b)) only contained the characteristic peaks of Si-O-Si. After the synthesis of RFMIPs (3B(c)), the characteristic peak of CDs can be clearly observed, which indicated the successful preparation of RFMIPs.

3.2.3 Thermogravimetric analysis

TGA evaluates the thermal stability of RFMIPs between 25-800 °C. As shown in Fig. 3C, when the temperature reached 200 °C, RFMIPs had a weight loss of about 6.3% in the first stage, which may be caused by evaporation of some organic solvents and physically adsorbed water. When the temperature exceeded 400 °C, the weight loss in the second stage started to accelerate, which may be because the imprinted lay er of RFMIPs started to degrade and reached stability at 600 °C, and the weight loss at this stage was about 10.4%. The experiments showed that SMMIPs had good thermal stability and usability in the range of 25-200°C.

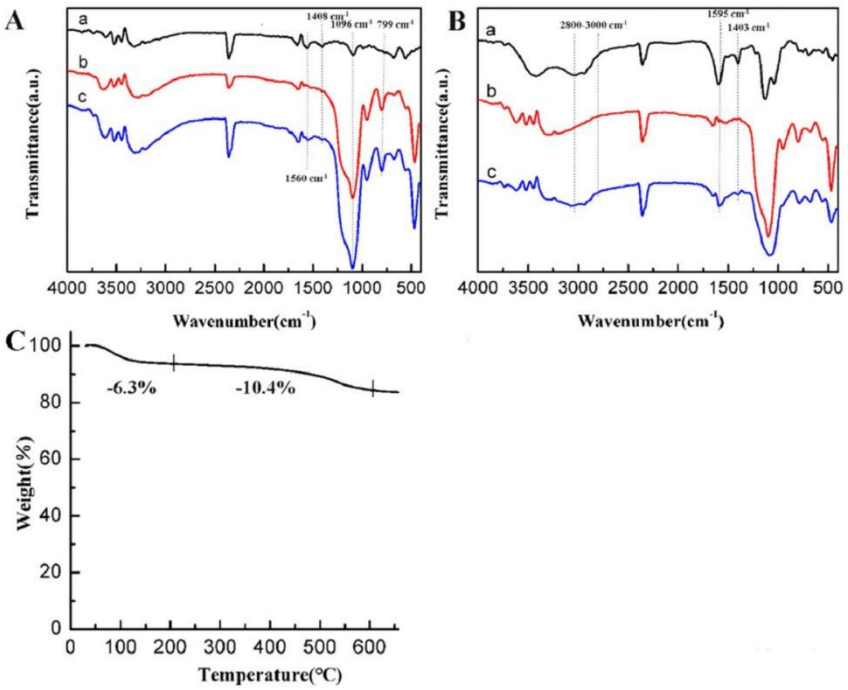

Fig. 3 (A) FT-IR spectrum of (a) CdTe, (b) SiO_2-NH_2 and (c) SiO_2@CdTe, (B) FT-IR spectrum of (a) CDs, (b) SS and (c) RFMIPs, (C) the thermogravimetric weight loss curve of RFMIPs

4. Conclusion

In this experiment, a combination of molecularly imprinted technology, epitope imprinting and nano-fluorescent materials was used to prepare RFMIPs, which was used for sensitive detection of nNOS. This method combines the selectivity and stability of an epitope-imprinted polymer with the sensitivity of ratio fluorescence. It could specifically recognize the template molecule nNOS.

References

[1] Fresco-Cala B, Batista AD, Cárdenas S. Molecularly imprinted polymer micro-and nano-particles: A review[J]. Molecules,2020,25(20):4740.

[2] Díaz-Álvarez M, Martín-Esteban A. Molecularly imprinted polymer-quantum dot materials in optical sensors: An overview of their synthesis and applications[J]. Biosensors,2021,11(3): 79.

[3] Saylan Y, Akgönüllü S, Yavuz H, et al. Molecularly imprinted polymer based sensors for medical applications[J]. Sensors,2019,19(6):1279.

[4] Hu Y, Miao ZY, Zhang XJ, et al. Preparation of microkernel-based mesoporous (SiO_2-CdTe-SiO_2)@SiO_2 fluorescent nanoparticles for imaging screening and enrichment of heat shock protein 90 inhibitors from Tripterygium Wilfordii [J]. Analytical Chemistry,2018,90(9):5678-5686.

[5] Liang AX, Hou HP, Tang SS, et al. An advanced molecularly imprinted electrochemical sensor for the highly sensitive and selective detection and determination of Human IgG[J]. Bioelectrochemistry,2021,137:107671.

[6] Taguchi H, Sunayama H, Takano E, et al. Preparation of molecularly imprinted polymers for the recognition of proteins via the generation of peptide-fragment binding sites by semi-covalent imprinting and enzymatic digestion[J]. Analyst, 2015,140(5):1448-1452.

[7] Raoul T, Anna T, Bianca S, et al. Recognition of protein biomarkers using epitope-mediated molecularly imprinted films:

Histidine or cysteine modified epitopes? [J] Biosensors and Bioelectronics, 2018,123:260-268.
[8] Wei JR, Ni YL, Zhang W, et al. Detection of glycoprotein through fluorescent boronic acid-based molecularly imprinted polymer[J]. Analytica Chimica Acta, 2017,960:110-116.
[9] Zhang Z, Li JH, Wang XY, et al. Quantum dots based mesoporous structured imprinting microspheres for the sensitive fluorescent detection of phycocyanin[J]. ACS Applied Materials & Interfaces, 2015,7(17):9118-9127.
[10] Jalili R, Amjadi M. Surface molecular imprinting on silane-functionalized carbon dots for selective recognition of nifedipine[J]. RSC Advances, 2015, 5(90): 74084- 74090.
[11] Zhang SH, Wen L, Yang JP, et al. Facile fabrication of dendritic mesoporous SiO_2@CdTe@SiO_2 fluorescent nanoparticles for bioimaging[J]. Particle & Particles Systems Characterization. 2016,33(5):261-270.