Highly sensitive fluorescence resonance energy transfer (FRET)-based nanosensor for rapid detection of clenbuterol

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Abstract
In this study we investigate the fabrication of a fluorescence resonance energy transfer (FRET)-based nanosensor for the detection of clenbuterol. The nanosensor consists of CdTe quantum dots coated by clenbuterol recognizable agent naphthol and diazotized clenbuterol. Changes in maximal photoluminescent intensities of the nanosensor were utilized to measure clenbuterol concentrations. The maximal photoluminescent intensities of the nanosensor were found to decrease with increasing clenbuterol concentrations, following a linear correlation. We have successfully fabricated a nanosensor for detection of clenbuterol with sensitivity up to 10 pg ml⁻¹.

Keywords: clenbuterol, FRET effect, quantum dots, nanosensor

Classification numbers: 4.01, 6.08

1. Introduction
Growth-promoting hormones such as clenbuterol are often used by the breeder 21 days before sales of farmed animals. These hormones are very strong and active agents. The use of growth-promoting hormones brings huge economic benefits for the breeders, but these hormones also have negative effects on human health. Unlike some toxic agents that decompose at high temperature, clenbuterol in pork meat cannot be decomposed by cooking at high temperature. The clenbuterol residuals could affect human health, causing diseases such as cancers, poisoning, heart failure, hypertension and even death [1–3]. Because of these side effects, the use of clenbuterol in the breeding industry has been prohibited all over the world since 2000. However, there is still illegal usage of clenbuterol, especially in pig and chicken breeding.

The clenbuterol residual in animal products can hardly be detected by chemical methods. In the laboratory, clenbuterol can be detected by methods such as enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC/MS). However these methods often require elaborating sample preparation procedures and involve tedious time-consuming protocols [4, 5]. As a result, these methods cannot be applied in breeding, aquaculture as well as in market control. Therefore, a lot of efforts have been made to develop nanosensors for quick detection of clenbuterol residuals with high sensitivity in animal products [6].

Recently, fluorescence resonance energy transfer (FRET) effect has been used for fabrication of nanosensor [7]. A FRET-based nanosensor can further improve the sensitivity of nanosensor. FRET concept is based on the energy transfer from donor excitation state to acceptor. Normally, donors have fluorescence emission at short wavelengths which overlaps with that of acceptors. The efficiency and speed of energy transfer depends on overlapping degrees of donor and acceptor fluorescence and on the quantum efficiency of donors as well as the distance between donors and acceptors [8, 9].

In this study we investigated the fabrication of FRET-based nanosensor with the aim to detect clenbuterol. CdTe quantum dots (QDs) were used as donors while acceptors were formed by diazotization of clenbuterol with...
2. Experimental

2.1. Materials

Clenbuterol with chemical formula C_{12}H_{18}Cl_{2}N_{2}O (molecular weight of 277.19 g mol^{-1}), the naphthol compound 2-amino 8-naphthol-6-sulfonic acid with the chemical formula C_{10}H_{9}O_{4}NS (molecular weight of 239 g mol^{-1}), mercaptoacetic acid (MPA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich company. All chemicals were analytical grade and used as received. CdTe quantum dots coated by MPA were kindly supplied by the Institute of Materials Science of VAST.

2.2. Fabrication of nanosensor and characterization methods

2.2.1. Preparation of super-molecules QDs and diazotized clenbuterol. The QDs coated by MPA contain carboxyl group (-COOH) on the surface and thus they are soluble in water. According to the literature [11], the functional groups on the surface of QDs such as amine, thiols or carboxy can be further modified with EDC. In this case, the carboxyl group on the surface of QDs was firstly activated by EDC and then could react with the amino group from 2-amino 8-naphthol-6-sulfonic acid to form stable amide coupling. Figure 1 shows the synthesis scheme of super-molecule QDs by modifying with 2-amino 8-naphthol-6-sulfonic acid. By these super-molecule QDs, the –SO_{3}H group could further increase the water solubility of QDs.

Figure 2 shows the scheme for preparation of diazotized clenbuterol [10]. The clenbuterol was diazotized by using NaNO_{3} in HCl medium at temperature of 10\degree C.

2.2.2. Preparation of sensor solution for detection of clenbuterol. The sensor solution was prepared as follows. Firstly, a stock solution (1 µg ml\(^{-1}\)) of diazotized clenbuterol was prepared by dissolving an appropriate amount of diazotized clenbuterol in phosphate buffer (pH = 7.4). The working solution with different clenbuterol concentration was prepared by dilution of stock solution and stored at 4\degree C until used.

Similarly, the solution of super-molecule QDs (1 µg ml\(^{-1}\)) was diluted in phosphate buffer (pH = 7.4) solution until a concentration of 0.1 µg ml\(^{-1}\) was achieved. Before using, the solution of super-molecule QDs was annealed at 4\degree C overnight.

Finally, the sensor solution was prepared by mixing of the super-molecule QDs solution and the solution of diazotized clenbuterol. The procedure for preparation of sensor solutions with different clenbuterol concentrations is presented in table 1.
Table 1. Procedure for preparation of nanosensor solution with different clenbuterol concentration.

| Sample | QDs solution (ml) | Diazo-Clen. solution (ml) | H₂O | Clen. concentration (pg ml⁻¹) |
|--------|-------------------|--------------------------|-----|-----------------------------|
| 1      | 1                 | 0                        | 1   | 0                           |
| 2      | 1                 | 0.25                     | 0.75| 10                          |
| 3      | 1                 | 0.5                      | 0.5 | 20                          |
| 4      | 1                 | 1                        | 0   | 40                          |

Figure 4. Working principle of nanosensor for detection of clenbuterol.

2.3. Working principle of nanosensor and characterization methods

By mixing the two solutions, super-molecule QDs and diazotized clenbuterol, the coupling reaction between diazotized clenbuterol and naphthol group on the surface of QDs occurs. Figure 3 shows the reaction scheme of the diazo group of diazotized clenbuterol with the naphthol compound on the surface of QDs.

This reaction forms the specific orange yellow color of azo compound. Besides, this reaction happens only when there is diazo group. Thus, this reaction can be used as specific reaction to recognize clenbuterol in solution. In the practical case, there are also some amine groups in protein and urea from a test sample obtained from urine or internal organs of animals but this amine group cannot form diazo coupling with QDs. In other words, this kind of nanosensor can recognize only the diazotized clenbuterol. Figure 4 shows the working principle of the nanosensor for detection of clenbuterol.

By absorption of light, the resonance energy transfer from QDs to clenbuterol occurs and the photoluminescent properties of the sensor can be changed by varying the clenbuterol concentration. In this case, changes in maximal photoluminescent intensity were utilized to indicate clenbuterol concentrations. Photoluminescence measurements were conducted on HR550 instrument (HORIBA JOBIN YVON) and transmission electron microscopy (TEM) images were taken on JEM-1010, Jeol at the Institute of Materials Science (VAST). UV–Vis spectra of the materials were recorded on GBC-285 instrument at the Institute of Chemistry (VAST).

3. Results and discussion

3.1. Morphology of super-molecule QDs

Figure 5 shows the TEM images of QDs coated by clenbuterol recognizable naphthol.

As reported by Liem et al [12], the as-prepared CdTe QDs are almost monodispersed spherical particles with diameters ranging from 3 to 5 nm. After coating with naphthol, diameters of the particles increase significantly, ranging from 19 nm to 30 nm. This result indicates that naphthol is successfully attached onto the surface of CdTe QDs.

Figure 5. TEM images of super-molecule QDs coated by clenbuterol recognizable naphthol.

Figure 6. UV–Vis and PL spectra of CdTe QDs [12].
3.2. Effect of naphthol on optical property of QDs

The super-molecule QDs has the core-shell structure and the outer shell is naphthol. The effect of naphthol on the optical property of QD was investigated. Figure 6 shows the UV–Vis spectrum and photoluminescence (PL) spectrum of QDs before modification with naphthol [12]. CdTe QDs have the maximum absorption peak at wavelength of 520 nm and the maximal emission peak at the wavelength of 585 nm.

Figure 7 shows the UV–Vis spectrum of naphthol. The maximal absorption peak of naphthol is about 281 nm and is out of the absorption range of CdTe QDs. This result...
Table 2. Procedure for preparation of organic dyes sensor solution with different clenbuterol concentrations.

| Sample | Naphthol solution (ml) | Diazo-Clen. Solution (ml) | H₂O (ml) | Clen. concentration (pg ml⁻¹) |
|--------|------------------------|---------------------------|----------|-----------------------------|
| 1      | 1                      | 0                         | 1        | 0                           |
| 2      | 1                      | 0.25                      | 0.75     | 10                          |
| 3      | 1                      | 0.5                       | 0.5      | 20                          |
| 4      | 1                      | 1                         | 0        | 40                          |

Figure 10. Photoluminescence of nanosensor at varying clenbuterol concentrations (samples 1, 2, 3, 4 = 0, 10, 20, 40 pg ml⁻¹, respectively).

Figure 11. Correlation between maximal photoluminescent intensities of nanosensor and clenbuterol concentrations.

means that naphthol has no effect on the absorption as well as emission of QDs.

Figure 8 shows the UV–Vis spectrum of clenbuterol. The maximal absorption peak of clenbuterol is about 301.5 nm.

Figure 9 compares the UV–Vis spectra of clenbuterol, diazotized clenbuterol and super-molecule QDs coupled with diazotized clenbuterol.

Comparing to the UV–Vis spectrum of clenbuterol, in the case of the diazotized clenbuterol there appears a new strong absorption peak at wavelength of 376.2 nm. This strong absorption peak of diazotized clenbuterol is assigned to the newly formed diazo group (–N=N–). In contrast, in the UV–Vis spectrum of the super-molecule QDs coupled with diazotized clenbuterol, the absorption peak of the diazo group of diazotized clenbuterol disappeared. This result clearly indicates that the coupling reaction between the super-molecule QD and the diazo group of the diazotized clenbuterol occurs and thus the absorption peak of the diazo group is absent.

Figure 12. Photoluminescence of organic dyes sensor at different clenbuterol concentrations (samples 1, 2, 3, 4 = 0, 10, 20, 40 pg ml⁻¹, respectively).

Figure 13. Correlation between maximal photoluminescent intensities of organic dyes sensor and clenbuterol concentrations.

3.3. Detection of clenbuterol by nanosensor

In this study the maximal photoluminescent intensity of the nanosensor was utilized to indicate clenbuterol concentrations. Figure 10 shows the photoluminescence of nanosensor at varying clenbuterol concentrations.

As shown in figure 10, the photoluminescence at different clenbuterol concentrations is not overlapped. The maximal photoluminescent intensities are found to be dependent on clenbuterol levels, decreasing with increasing concentrations. This means that the clenbuterol has a fluorescent quenching effect in the nanosensor. This result also indicates that there is quantum fluorescent energy transfer from QD to clenbuterol. With increasing clenbuterol concentrations, energy absorption by QDs also increases, and therefore the maximal photoluminescent intensity of nanosensor decreases.

Figure 11 shows that there is a linear correlation between the clenbuterol concentrations and the nanosensor photoluminescent intensities. The result clearly demonstrates the viability to determine clenbuterol concentrations based on this linear correlation.

3.4. Effect of QDs on the sensitivity of nanosensor

To investigate the effect of QDs on the sensitivity of the sensor, organic dyes sensor containing only naphthol compound coupled with diazotized clenbuterol were prepared. The procedures for the preparation of this kind of organic dyes sensor solution are shown on the table 2.
Figure 12 shows the photoluminescence of organic dyes sensor at different clenbuterol concentrations. Sample 1 is the photoluminescence of naphthol compound. Samples 2, 3 and 4 are the photoluminescence spectra of organic dyes sensor solutions with the clenbuterol concentration of 10, 20 and 40 pg ml$^{-1}$, respectively. As shown, the photoluminescence spectra of the organic dyes sensor at different clenbuterol concentrations are overlapped. The maximal photoluminescent intensity is almost unchanged at varying clenbuterol concentrations (see figure 13).

This result indicates that the QDs have a strong effect on the sensitivity of the nanosensor for the detection of clenbuterol. The quantitative determination of the clenbuterol concentration in the solution is not possible with organic dyes sensor without QDs. The result also confirms the importance of FRET effect for the detection of clenbuterol.

4. Conclusions

In this study we have successfully fabricated the FRET-based nanosensor for the detection of clenbuterol. The FRET-based nanosensor consists of super-molecule CdTe QDs coated with naphthol coupling with the diazotized clenbuterol. The maximal photoluminescent intensities of the nanosensor are found to decrease with increasing clenbuterol concentrations. Furthermore, there is a linear correlation between intensities and clenbuterol concentrations. In the absence of QDs in the organic dyes sensor, the quantitative clenbuterol concentration in solution cannot be determined. The reason is the lack of the FRET effect in the sensor.

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