Rapid and sensitive detection of clenbuterol using a fluorescence nanosensor based on diazo coupling mechanism

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Abstract
In this paper, the fluorescence resonance energy transfer (FRET) effect has been used for fabrication of nanosensor for the detection of clenbuterol. In the nanosensor, the CdTe quantum dots (QDs) are the donors while the acceptor is the super-macromolecule formed by the diazoation coupling mechanism between diazo clenbuterol and naphthylethylene diamine. Changes in fluorescence intensities of nanosensor were used to determine the clenbuterol concentration. We have successfully fabricated a nanosensor for detection of clenbuterol sensible to clenbuterol concentration of 10⁻¹² g ml⁻¹.

Keywords: clenbuterol, quantum dots, nanosensor, FRET, fluorescence

Classification numbers: 4.01, 6.08

1. Introduction

Clenbuterol (4-amino-α[(tert-butylamino)methyl]-3, 5-dichlorobenzyl alcohol hydrochloride) is a β-agonist, which can increase growth rate, accelerate protein development and reduce fat when used in the breeding industry. However, clenbuterol residues in meat are harmful to human health, causing diseases such as cancers, poisoning and heart failure [1]. Because of these side effects, the use of clenbuterol in the breeding industry has been prohibited all over the world since 2000. However, in Vietnam there is still illegal usage of clenbuterol, especially in pig and chicken breeding.

Fluorescence resonance energy transfer (FRET) first explored in the 1920s is increasingly gaining the attention of multi-disciplinary researchers [2]. It is a technique widely used in probing the conformational changes of biomolecules, examining the structural constitution of a targeted cell and monitoring intracellular processes and many others in vivo in biological applications. FRET is a non-radiative energy transfer from the excited state of a donor to an acceptor. FRET occurs when the emission spectrum of a fluorescent donor overlaps the absorption spectrum of an acceptor [3, 4]. As a result, the donor lifetime is shortened and the acceptor fluorescence is sensitized. The distance between the donor and acceptor has to be in the range of 1–10 nm for FRET to occur [2]. The main attraction of FRET is its sensitivity to very small spatial changes typically in the nanometer range.

Quantum dots (QDs) are semiconductor nanoparticles with very interesting optical properties, such as high quantum yield or narrow and size and tunable fluorescence spectrum [5–7]. To date, many kinds of QDs with different size, shape, and composition have been extensively investigated not only for their theoretical fundamentals but also for their practical applications, including in solar cells, optoelectronic transistor components, and fluorescent biological labels [8]. Among various QDs, cadmium telluride (CdTe) QDs have been widely used in industrial and biomedical applications because of their tunable photoluminescence (PL) within the visible range when excited by a single excitation wavelength. For instance, CdTe QDs are believed to be promising probes in the bio-imaging of living cells because of their many advantages such as higher photostability, greater...
controllability and narrower emission bands, and higher quantum yield in comparison with conventional fluorescent dyes [9].

In this paper we investigate the fabrication of a FRET based nanosensor using quantum dots as donor for detection of toxic residual chemicals such as clenbuterol in food. Changes in fluorescence intensities of sensor were applied to measure the clenbuterol concentration.

2. Experimental

2.1. Materials

Clenbuterol with chemical formula C12H18Cl2N2O (molecular weight of 277.19 g mol\(^{-1}\)), N-1-naphthylethylene diamine dihydrochloride with chemical structure C12H14N2.2HCl, sodium nitrite (NaNO2), hydrochloride acid were purchased from Merck. All chemicals were analytical grade and used as-received. CdTe quantum dots coated with 3-mercaptopropionic acid (MPA) were kindly supplied by Institute of Materials Science (IMS), Vietnam Academy Science and Technology (VAST).

2.2. Fabrication of nanosensor and characterization methods

2.2.1. Diazotization of clenbuterol. The diazotization of clenbuterol (DCL) was prepared in the same way as our previous work [10]. 3 mg clenbuterol and 3 ml HCl 0.01 M was placed in a flask and shaken. The flask was then kept in ice bath at a temperature of 0–5 °C in the dark. Then, 2 ml NaNO2 0.01 M was added dropwise to the flask. This mixture was stirred vigorously and allowed to remain for five minutes. Figure 1 shows the diazotization of clenbuterol.

2.2.2. Preparation of sensor solution for the 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 solutions with different clenbuterol concentrations were prepared by dilution of stock solution and stored at 4 °C until being used.

For each nanosensor sample, 1 μmol QDs and 10 mmol naphthylethylene diamine were injected into a vial. The reaction solution was incubated at 25 °C for 30 min with gentle shaking. By this process, the QDs with the carboxyl group (COOH) on the surface will form super-molecule QDs by attaching the naphthylethylene diamine on the surface through the electrostatic interaction between the negative charge of COOH group and the positive charge of the amine group of naphthylethylene diamine. Finally, the sensor solution was prepared by mixing of the super-molecule Qds solution and solution of diazotized clenbuterol.

2.2.3. Characterization methods. The UV-Vis absorption spectra of the samples were recorded by using spectrometer SP-3000 at Institute of Chemistry (IoC) VAST. The PL measurements were conducted on HR550 instrument (HORIBA JOBIN YVON) at IMS, VAST. The FTIR spectra of the samples were obtained by using an Impact 410 NICOLET at IoC, VAST.

3. Results and discussions

3.1. Working mechanism and FRET effect in the nanosensor

Figure 2 shows the working mechanism of the nanosensor for the detection of clenbuterol.

In this work naphthylethylene diamine was used as ligand which functions both in detecting of clenbuterol and in changing of the optical properties of the acceptor in order to fulfill the condition for FRET effect to occur in the nanosensor. As compared to our previous work [10] using 2-amino 8-naphthol-6-sulfonic acid for these functions, the immobilization of naphthylethylene diamine on QDs surface through physical bonding is easier than that of 2-amino 8-naphthol-6-sulfonic acid through chemical bonding (amide coupling reaction). In this nanosensor, the specific reaction for detection of clenbuterol is the coupling reaction between the diazo group of diazotized clenbuterol and the aromatic ring of naphthylethylene diamine. To confirm the coupling reaction, a reaction between diazo-clenbuterol and naphthylethylene
diamine was conducted and the azo products (NDCL) formed by this reaction was analyzed by FTIR method (figure 3).

On table 1 is the result of FTIR analysis of the azo products.

The FTIR analysis result indicates that the formation of the azo functional group (–N=N–) at the wave number 1576 cm\(^{-1}\) is clearly observed [11].

The coupling reaction between the diazo group of diazotized clenbuterol and the aromatic ring of naphthylethylene diamine could be reconfirmed by the analysis of UV-Vis spectra of pristine clenbuterol, diazo-clenbuterol and diazo-clenbuterol coupling with naphthylethylene diamine (figure 4).

Pristine clenbuterol has the maximal absorption peak at the wavelength of 294 nm. By the diazotization of clenbuterol, the maximal absorption peak of clenbuterol shifted to longer wavelength at 345 nm. This peak of diazotized clenbuterol reveals the newly formed diazo group (–N≡N–). In the case of diazotized clenbuterol coupling with naphthylethylene diamine, there is a new strong absorption peak at wavelength of 464 nm and the absorption peak of the diazo group of diazotized clenbuterol has disappeared. This result demonstrates that the coupling reaction between naphthylethylene diamine and the diazo group of the diazotized clenbuterol is successful so the absorption peak of the diazo group is absent.

To investigate the FRET effect in the nanosensor, figure 5 shows the UV-Vis absorption spectrum of diazo-clenbuterol coupling with naphthylethylene diamine as acceptor and the photoluminescent spectrum of QDs CdTe as donor.

There is an overlapping between the absorption spectrum of the acceptor and the photoluminescent spectrum of the donor. This result means that the energy from excitation state of QDs from 450 nm to 600 nm could transfer to the acceptor and cause fluorescence quenching of the donor. In other words the FRET effect actually occurred in the nanosensor. Therefore, a decrease in the emission intensity of QDs CdTe can be used to quantify the clenbuterol concentrations. The FRET effect in the nanosensor is the most important factor to enhance the sensibility as well as to quantify the clenbuterol concentrations. Nanosensor without QDs as donor or without the FRET effect cannot identify the change of clenbuterol concentration, as reported in our previous work [10].

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**Table 1. FTIR analysis of azo product.**

| Wavenumber (cm\(^{-1}\)) | Bonding                  |
|--------------------------|--------------------------|
| 3332                     | N-H stretching           |
| 2976                     | C-H stretching           |
| 1625                     | C=C aromatic ring stretching |
| 1576                     | –N=N– stretching         |
| 1197                     | C-N stretching           |
| 768                      | C-Cl stretching          |

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**Figure 3.** FTIR spectrum of azo product formed by reaction between diazo-clenbuterol and naphthylethylene diamine.

**Figure 4.** UV-Vis spectrum of clenbuterol (1), diazo clenbuterol (2), diazo clenbuterol coupling naphthylethylene diamine (3).

**Figure 5.** Overlapping spectrum of donor’s fluorescence and acceptor’s absorption spectrum.
3.2. Detection of clenbuterol at different concentrations by nanosensor

Figure 6 shows the PL spectrum of QDs CdTe and PL spectra of sensor solution at different clenbuterol concentrations.

Table 2 shows the values of the PL intensity of the nanosensor corresponding to the clenbuterol concentrations.

As can be seen from figure 6(a), well-defined maximal emission peaks were observed at PL spectra of the nanosensors and the maximal emission peak intensity decreased with the increasing of target concentrations. At the clenbuterol concentration of \(10^{-13}\) g ml\(^{-1}\), the form of the PL spectrum of the nanosensor is almost identical to the PL spectrum of QDs CdTe. That means that the concentration limit for detection of clenbuterol by using this nanosensor is \(10^{-12}\) g ml\(^{-1}\). In contrast, at the clenbuterol concentration of \(10^{-4}\) g ml\(^{-1}\) a very broad emission peak was observed. That indicates that the emission of QDs CdTe was fully quenched at the clenbuterol concentration higher than \(10^{-4}\) g ml\(^{-1}\).

For quantitative determination of clenbuterol concentrations, figure 6(b) shows the correlation between the PL intensity of nanosensor and the negative logarithmic clenbuterol concentrations. A good linearity of the relationship was observed at the wide clenbuterol concentration range from \(10^{-7}\) g ml\(^{-1}\) to \(10^{-12}\) g ml\(^{-1}\). These results will open a new way for control and detection of clenbuterol residual in Vietnam.

4. Conclusions

A fluorescence nanosensor based on diazo coupling mechanism was successfully fabricated using FRET effect and first developed for rapid, sensitive and easy quantitative detection of a trace amount of clenbuterol. The modification of QDs’ surface via naphthylethylene diamine through electrostatic interaction as well as the diazo-coupling reaction between the diazotized clenbuterol and the aromatic ring of naphthylethylene diamine by fabrication of the nanosensors were fast and uncomplicated. It was shown that the sensitivity of the nanosensor for the detection of clenbuterol was determined for clenbuterol concentration as low as \(10^{-12}\) g ml\(^{-1}\). A good linearity of the relationship between the PL intensity and clenbuterol concentration was observed at the clenbuterol concentration range \(10^{-7}\) g ml\(^{-1}\) to \(10^{-12}\) g ml\(^{-1}\). These results will open a new way for control and detection of clenbuterol residual in Vietnam.

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