Biosensors and Bioelectronics 24 (2009) 2051–2056

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Ultrasensitive immunoassay of 7-aminoclonazepam in human urine based on CdTe nanoparticle bioconjugations by fabricated microfluidic chip

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ARTICLE INFO

Article history:
Received 26 July 2008
Received in revised form 7 October 2008
Accepted 21 October 2008
Available online 31 October 2008

Keywords:
Ultrasensitive
Immunoassay
7-Aminoclonazepam
CdTe nanoparticle
Microfluidic Chip
LC/MS/MS

ABSTRACT

The present paper described a rapid and ultrasensitive detection method using a microfluidic chip for analyzing 7-aminoclonazepam (7-ACZP) residues in human urine. A microfluidic chip-based immunoassay with laser-induced fluorescence (LIF) detection based on the water-soluble denatured bovine serum albumin (dBSA)-coated CdTe quantum dots (QDs) was prepared for the ultrasensitive detection of 7-ACZP. The whole procedure including the chip and the control software was designed and constructed in our own laboratory. The detection of 7-ACZP could be completed within 5 min. The results demonstrated that under the optima conditions, 7-ACZP residues could be detected with a precision of 5% relative standard deviation (RSD), and the linear range and the limit of detection (LOD) for 7-ACZP were 1.1–60.1 and 0.021 ng mL^{-1}, respectively. This method was compared with ELISA and showed a good correlation. This microfluidic chip with LIF detection was applied to the determination of 7-ACZP residues in positive human urine samples, and the results were confirmed by high-performance liquid chromatography and tandem mass spectrometry (LC/MS/MS). This ultrasensitive detection technique was proved to be practical for clinical use.

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1. Introduction

Since the development of microfluidic analytical technology in the last century, much progress has been made in this field, and some of the technologies have been industrialized. Recently some reports have indicated that microfluidic or lab-on-a-chip devices are promising platforms to achieve rapid and sensitive immunological detection of bacteria and proteins (Harrison et al., 1993; Liu et al., 2005). But the actual application of this technology in the routine detection of drug residues has rarely been studied.

Benzodiazepine is a type of psychotropic drug, which can eliminate erethism, manic anxiety. Benzodiazepines have a calming effect on patients by selectively inhibiting unrest and tension. In general, benzodiazepines act as hypnotics in high doses, anxiolytics in moderate doses, and sedatives in low doses (Ashton, 1994). In China, with the ever-increasing pressure of work, the situation of young people suffering from insomnia is more and more serious. Benzodiazepines are mainly used in the treatment of insomnia in clinical cases. Clonazepam (CZP) is a benzodiazepine derivative (Elian, 2003; Salamone, 2001) and 7-aminoclonazepam (7-ACZP) is a major urinary metabolite (target metabolite) of clonazepam.

Although there are many published protocols such as HPLC, GC–MS methods which can be used in the detection of drug residues in clinical cases, these methods are all laborious, time consuming and costly (Kurisaki et al., 2005; Miller et al., 2006; Wolff et al., 1997; Zhu et al., 2003; Smink et al., 2004, 2006; Quintela et al., 2006; Hegstad et al., 2006; Øiestad et al., 2007; Borrey et al., 2002). Our group has also tried methods based on ELISA, CE and so on for the detection of 7-ACZP (Peng et al., 2008; Li et al., 2008), however, we found that these methods were unable meet the requirements of high efficiency, ultrasensitivity, and rapid determination. It is significant challenge to develop a rapid, sensitivity, simple assay for detecting 7-ACZP in clinical cases.

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doi:10.1016/j.bios.2008.10.015

Quantum dots (QDs) have the potential of becoming a new type of fluorescent probe for many biological and biomedical applications (Chan and Nie, 1998; Bao et al., 2004; Xing et al., 2007; Wang et al., 2002; Hua et al., 2006; Wu et al., 2007; Kermana et al., 2007; Ma et al., 2007; Megan et al., 2005). As fluorescent probes, QDs have several advantages over conventional organic dyes. Their emission spectra are narrow, symmetrical, and tunable according to their size and material composition, allowing closer spacing of different probes without substantial spectral overlap. They exhibit excellent photo stability. They also display broad absorption spectra, making it possible to use a range of light sources and to minimize sample autofluorescence by choosing an appropriate excitation wavelength.

In this study, an easy microfluidic chip device with the laser-induced fluorescence detection was developed in our laboratory. The present paper describes, for the first time to our knowledge, a rapid and ultrasensitive detection method using microfluidic chips for analyzing 7-ACZP residues. Our device was fabricated based on the water-soluble denatured bovine serum albumin (dBSA)-coated CdTe quantum dots (QDs) labeled antibody and it was used to develop a new ultrasensitive detection method for the determination of 7-ACZP residues in clinical samples. The detection assay took less than 5 min for each sample, and all reagents used were economical, in comparison with other methods such as GC–MS and HPLC. This new method was also compared with ELISA and confirmed by LC/MS/MS. Validation of the microfluidic chip in the detection of 7-ACZP residues in human urine samples made it possible to apply this technology to the detection of other drug residues in clinical samples as a routine protocol.

2. Materials and methods

2.1. Chemicals

All the chemicals were of analytical grade or better, and ultrapure water (18 MΩ), purified on a Water PRO PS system (Labconco, Kansas city, MO, USA), was used for the preparation of all the aqueous solutions. Poly (dimethylsiloxane) (PDMS) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), ovalbumin (OVA) and diaminodiphenylmethane (DDM) were purchased from Sigma (USA). 7-Aminoclonzepam and 7-aminonitrazepam were purchased from Cerilliant (USA). The anti-7-ACZP antibody was produced in our laboratory. The electrophoresis buffer was 5 mmol L^{-1} Tris buffer (pH 9.3). All solutions used in the experiment were filtered through 0.22-μm membrane filters (Costar, Cambridge, MN, USA) before use.

2.2. Design and fabrication of microfluidic devices

The high and low voltage generators were bought from Shanghai Instrument Co. Ltd. (Shanghai, China). An Argon laser (488 nm) solid laser generator (20 mW, 85BCD-020-230, Melles Griot Inc.) was set up as the light source for the LIF. The features and fabrication of the microfluidic devices used here were shown in Fig. 1. The whole microfluidic device was as large as the computer host. A diagram of the microchip (63.5 mm × 31.75 mm) design was shown in Fig. 1, and the modified procedure of this fabricated device used was as follows. Briefly, the channels which were 20 μm deep and 100 μm wide were arranged in a cross formation on the chip. The inner surface of the channel was modified with DDM which was allowed to solidify for 5–10 min before each detection assay. The adopted optimum separation voltage between the sample and waste pore was 800 V for 30 s and the separation voltage between the buffer and buffer waste pore was 1.8 kV.

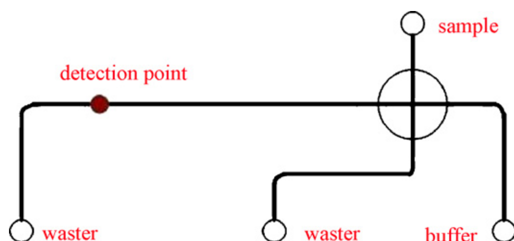

Fig. 1. Microfluidic chip with LIF detection device system is shown in the schematic diagram of the chip.

The interior construction and exterior shape of microfluidic chip device were shown in the Supporting information in Appendix B. The control software for the device was written in our laboratory with VC++, and it was displayed in Supporting information.

2.3. Preparation of denatured BSA-coated QDs

The synthesis and characterization of CdTe QDs is according to the modified reference methods (Bao et al., 2004). The detailed process is as follows: Tellurium powder was chosen as a starting material to prepare NaHTe aqueous solution. Briefly, it was reduced by slightly excessive sodium borohydride in water in an ice-bath. After Te was completely reduced, 0.5 M H_2SO_4 was introduced to generate H_2Te gas that was discharged by a N_2 flow into another flask containing 0.05 M NaOH aqueous solution. Finally, an aqueous solution of 0.05 M NaHTe was obtained. Then a certain volume of the NaHTe solution was injected into a $CdCl_2$ solution containing thioglycolic acid (TGA). The pH value of latter solution was fixed at 11.2. The molar ratio of Cd^{2+}/Te^{2-}/RSH was set to 1:0.5:2.4. The reaction between cadmium ions and NaHTe took place immediately after the injection of NaHTe solution and changed the mixture from colorless to orange. Then the orange solution was heated till boiling. TGA-stabilized CdTe nanocrystals were obtained. The sizes of the CdTe QDs exhibiting different fluorescence were controlled by the reflux time. The concentration of the QDs is referring to the Cd^{2+} according to calculation of the added $CdCl_2$.

BSA (16.5 mg) was dissolved in 10 mL deionized water, and then 0.42 mg $NaBH_4$ was added to the BSA solution with stirring. After being stirred for 1 h at room temperature, the solution was heated to 60–80 °C in a water bath for 15 min until no more gas (H_2) was produced. BSA was denatured and most of its disulfide bonds were converted to sulfhydryl groups. The final concentration of the dBSA aqueous solution was 5×10^{-5} mol L^{-1}. The solution of quantum dots (QDs) was mixed with the dBSA solution in different molar ratios (1:1,1:2,1:4,1:6). The mixture was heated to 60–80 °C in a water bath for 15 min. The solution was then incubated at room temperature for 2 days (Xing et al., 2007). The results were identified by SDS-PAGE and fluorescence scan. A 10% separating gel and 4% stacking gel was used for SDS-PAGE separation.

2.4. Conjugation of dBSA QDs with anti-7-ACZP antibody

The dBSA-coated QDs were further modified with succinic anhydride in order to convert the primary amine groups of the dBSA into carboxyl groups using the following procedures (Wu et al., 2007; Kermana et al., 2007; Ma et al., 2007; Megan et al., 2005): dBSA-coated QDs were transferred to 0.1 M phosphate buffer (PB), pH 7.4. Solid succinic anhydride was added in three portions. A molar ratio of 20:1 of succinic anhydride with respect to the sum of lysine and tyrosine residues of BSA was used. During the reaction, the pH was maintained between 7.5 and 8.5 with NaOH (0.1 M). After the pH of the solution was stabilized at 8.0 for 1 h, the resulting solution

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