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Chemiluminescent detection integrated with microdialysis sampling for label-free measuring the affinity of ractopamine monoclonal antibody

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ABSTRACT

A novel label-free protocol was developed for measuring the affinity between ractopamine and its monoclonal antibody (McAb) based on microdialysis (MD) on-line sampling integrated with flow injection chemiluminescent detection. In this study, unbound ractopamine was sampled by MD probe from homogeneous immunoreaction equilibrious systems, and then real-time quantified using flow injection chemiluminescent detection. The quantified concentrations of unbound ractopamine in the immunoreaction equilibrious systems were treated with Scatchard analysis and Klotz analysis to obtain the affinity constant. The mean recovery of MD probe for sampling ractopamine was found to be 24.2%. The affinity constants calculated by Scatchard analysis and Klotz analysis both were $1.0 \times 10^6\ M^{-1}$, indicating that the investigated ractopamine mouse McAb was a medium-affinity antibody. The result showed good agreement with that obtained from thiocyanate elution test. This protocol for measuring antibody affinity is free of protein conjugation of hapten and enzyme labeling of McAb. Therefore it avoids affinity decrease resulting from steric hindrance, occupancy of the antigenic determinants, and deactivation of antibody, which has been frequently encountered in the reported conventional approaches. It opens up a new pathway for direct measurement of antibody affinity with a facile, rapid, accurate and low-cost approach.

1. Introduction

Ractopamine is a β_2-agonist agent that has been used to improve lean meat percentage and feed conversion rate by reducing fat deposition and promoting protein accretion in animal husbandry [1–4]. However, it is frequently reported that the additive for food-producing animals poses potential hazards to human health [5–7]. Thus ractopamine has been rigorously banned to be used as growth promoter in many countries including China, European Union and Russia [8–10]. Immunoassay has been widely used for rapid screening of this agent in animal products, and thus it is essential to prepare ractopamine monoclonal antibody (McAb) [11–13]. In order to prepare and screen McAb with ideal performance, one of the most vital works is to measure the affinity of McAbs from various hybridoma cell lines [14–17].

To date, the mostly reported approach for measuring McAb affinity is based on enzyme labeling mode. This mode usually applies signal enzyme to label McAb, and then measures the antibody affinity utilizing heterogeneous enzyme-linked immunosorbent assay. The methods based on this mode include competitive binding assay [18,19], non-competitive enzyme immunoassay [20,21] and thiocyanate elution test [22,23]. Obviously, this mode requires rather long time and skilled personnel for enzyme labeling of McAb [24]. Furthermore, the chemical reactions for enzyme labeling sometimes cause deactivation of McAb and often lead to decrease of McAb affinity [25].

Besides the above mentioned enzyme labeling-based methods, some label-free protocols including quartz crystal microbalance [26] and surface plasmon resonance [27] can also be applied to measure McAb affinity because frequency shift and refractive index reflect the immunological binding events occurring on the sensors. These approaches require immobilizing antigen/hapten onto the sensor surface prior to measuring the affinity of McAb since a heterogeneous format is adopted. Nevertheless, haptens cannot be effectively immobilized due to their very low molecular weight. Thus they are usually conjugated with carrier protein to obtain artificial antigens with much higher molecular weight before use. However, some haptens are not fit for protein conjugation owing to the unactive chemical structures, hence it is usually demanded to prepare their structural analogs possessing active functional groups. Moreover, the protein conjugation might lower their binding ability to McAbs due to the steric hindrance or occupancy of the antigenic determinants.

Microdialysis (MD) is a sampling technique for continuously monitoring of analytes with biological interest both *in vivo* and *in vitro* [28,29]. Small-molecular substances passively diffuse into the MD probe due to the concentration gradient, and then are carried by the slowly moving perfusate into a sample collection vial or analysis system,

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while macromolecular substances such as cells and proteins can be effectively excluded. Therefore, MD can be used as both a sampling technique and a clean-up procedure. To minimize solution evaporation and shorten analytical time, MD system can be directly integrated with some instrumental analytical techniques such as liquid chromatography [30] and capillary electrophoresis [31]. Flow injection (FI)-chemiluminescence (CL) is a promising automated analytical technique, which shows a series of advantages such as minimal manual manipulation, real-time detection, ideal repeatability and high throughput [32]. Furthermore, FI coupling with MD can also minimize solution evaporation resulted from the very minor sampling amount.

Herein, we developed a novel label-free protocol for measuring the affinity of ractopamine McAb using MD sampling integrated with FI-CL detection. After ractopamine McAb was incubated with its hapten in a homogeneous system, the unbound ractopamine was sampled by a MD probe and carried into a FI-CL system for quantification. Then the affinity of ractopamine mouse McAb was obtained using Scatchard analysis and Klotz analysis.

2. Experimental

2.1. Reagents and Materials

Ractopamine mouse McAb and ractopamine-conjugated bovine serum albumin were both purchased from Lvdu Bio-Sciences and Technology Co., Ltd. (China). According to the information provided by the manufacturer, the molecular weight of the mouse McAb was 65 KDa. Ractopamine hydrochloride was provided by Guangzhou Ucando Biotechnology Co., Ltd. (China). Horseradish peroxidase-labeled goat anti-mouse IgG and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution were both obtained from Bioss Biotechnology Co., Ltd. (China). Luminol was supplied by Sigma-Aldrich (USA). All other chemicals were of analytical reagent grade and utilized without further purification. Ultrapure water (18.2 MΩ) treated by an ELGA water purification system (France) was applied to prepare all aqueous solutions. Polystyrene high-affinity 96-well microplates were provided by Greiner Bio-One Biochemical Co., Ltd. (Germany).

The antibody dilution buffer and the MD perfusate both were phosphate buffered saline (PBS) at 0.010 M and pH 7.4. The microplates coating buffer was carbonate buffered saline at 0.10 M and pH 9.0. The washing buffer was PBS containing 0.05% Tween-20. SuperBlock® T20 (Thermo Fisher Scientific Inc., USA) was used as the blocking buffer. A luminol solution (2.5×10^{-6} M) prepared in 100 mM NaOH was applied as CL reagent solution 1, while a $KMnO_4$ solution (1.0×10^{-5} M) dissolved in ultrapure water was applied as CL reagent solution 2.

2.2. Apparatus32

The MD sampling system consisted of a CMA 20 Elite MD probe (CMA Microdialysis Inc., Sweden) and a syringe pump (Bioanalytical Systems Inc., USA) equipped with a 1000-μL syringe. The MD probe (schematically illustrated in Fig. 1A) was constructed by a polyarylethersulfone hollow semipermeable membrane with a length of 10 mm and a MWCO of 20 kDa, an inlet tubing (blue) and an outlet tubing (grey). The inlet tubing and the outlet tubing were linked with the syringe pump and the injection valve, respectively.

As illustrated in Fig. 1B, FI-CL detection was performed using an IFFM-E FI-CL analyzer (Xi'an Remax Electronic Science & Technology Co., Ltd., China). The whole system comprised two peristaltic pumps for solution delivery, an injection valve for sample loading and injection, a flow cell and an optical detector. Teflon tubings with an inner diameter of 0.25 mm were utilized to connect all flow-through components of the whole FI system. The absorbance measurements for thiocyanate elution tests were performed on an Infinite® 200 PRO microplate reader (Tecan, Austria).

2.3. Quantification of Ractopamine Using FI-CL System

With the injection valve kept on the loading position, the carrier stream (water) and the CL reagent solutions were delivered using the peristaltic pumps at a flow rate of 2.2 mL min^{-1} to obtain a stable baseline. Then the CL signal for ractopamine quantification was measured by injecting 30 μL of sample solution into the carrier stream by switching the valve to the injection position. Thus the sample solution merged with the mixture of CL reagent solutions in the flow cell, and produced CL emission for quantification.

2.4. Procedure of MD Sampling

To sample ractopamine, a MD probe was dipped into 1.0 mL of sample solution that was constantly stirred and maintained at 37 °C. The sampling of ractopamine was performed by perfusing the MD probe with PBS at a rate of 2.5 μL min^{-1} through the semipermeable membrane. Then the effused dialysate was introduced into the injection valve of the FI system to perform CL detection.

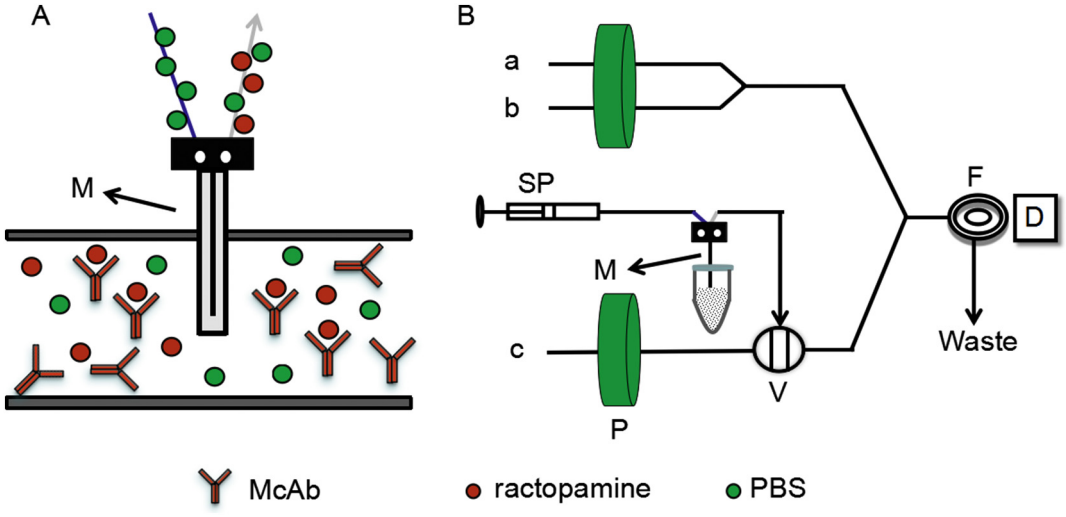

Fig. 1. (A) Principle of MD sampling of ractopamine. (B) Schematic diagram of measuring the affinity of ractopamine McAb using MD on-line sampling integrated with FI-CL detection: (SP) syringe pump, (M) MD probe, (P) peristaltic pump, (V) injection valve, (F) flow cell, (D) optical detector, (a) luminol (b) $KMnO_4$, (c) water carrier stream. (For interpretation of the references to colour in this figure, the reader is referred to the online version of this chapter.)