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Ultra-sensitive Determination of Salinomycin in Serum Using ICP-MS with Nanoparticles

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An ultra-sensitive detection method for small molecules such as antibiotics was developed using ICP-MS with magnetic and TiO_2 nanoparticles. Since most of the antibiotics are too small to employ a sandwich-type extraction through an immunoreaction, a non-specific platform was employed, in which the target was extracted by magnetic separation, followed by tagging with TiO_2 nanoparticles of 11.2 nm for ICP-MS measurement. The detection limit for salinomycin obtained from spiked serum samples was 0.4 ag mL^{-1} (± 10.3%), which was about 1.5×10^6 times lower than that of LC-MS/MS and about 1.2×10^{11} times better than that of ELISA. Such an excellent sensitivity enabled us to study the toxicity of antibiotics exposed to human beings by determining them in serum.

Key Words : Bioanalysis, Antibiotic detection, Particle tagging, ICP-MS, Nanoparticles

Introduction

Element tagging along with ICP-MS gave us a promise to determine various bio targets with multiplex detection.[1,2] Recently developed particle tagging can substitute it rapidly because of high stability and sensitivity owing to the signal amplification of nanoparticles in ICP-MS measurement. However, the particle tagging has still some issues remained, such as proper particle size, surface modification, and bio compatibility, particularly geometrical limitation of tagging for small molecules like antibiotics. Experimentally, the similar particle size to the target molecules were preferred in order to achieve minimum particle loss and better stability in magnetic separation. And it can also improve the sensitivity through the optimized binding ratio. As a typical example, a sandwich-type treatment platform using nanoparticles of near 100 nm reported excellent analytical performance to determine a biomarker using ICP-MS.[3] However, unlike typical biomarkers (28 kDa-200 kDa), small molecules like antibiotics are geometrically too small to be sandwiched. So far, no report has been issued for the determination of small antibiotics using ICP-MS with nanoparticle tagging.

Now, we developed an analytical method to determine antibiotics using ICP-MS with TiO_2 nanoparticles. For this, salimomycin in serum was selected for demonstration. Salinomycin is one of antibiotics, treating coccidial infection of poultry and exhibiting the activity against mycobacteria.[4,5] It improved the effectiveness of feed and growth facilitation for a ruminant and livestock.[6] Intoxicated accidents due to the misuse of salinomycin were reported recently, which caused various clinical signs to human beings or animals such as nausea, diarrhea, vomiting, *etc*.[7-9] Since EU prohibited the use of salinomycin officially, the ultrasensitive detection in food and serum was needed for the study of pharmacokinetics and human toxicity.[10] Various analytical methods to determine salinomycin were developed so far, such as enzyme-linked immunosorbent assay (ELISA)[11] and chromatography-hyphenated mass spectrometry.[12-16] The organic mass spectrometry showed high resolution and sensitivity with the detection limit of 0.01 μg kg^{-1}. ELISA offers fast analysis but it suffers from relatively poor detection limit in the range of 50 to 300 μg kg^{-1}.

In this work, we employed a non-specific platform for salinomycin determination, in which the target was non-specifically bound to the magnetic nanoparticles (MNPs) for magnetic separation and tagged by probe nanoparticles for signal amplification in ICP-MS measurement. The magnetic nanoparticles and TiO_2 nanoparticles in the size of ~10 nm were synthesized and modified for extraction and tagging. In particular, TiO_2 nanoparticles were chosen as the probes in this demonstration because they can be easily modified with amine groups through the formation of silica shell. Compared to the classical techniques, the developed analytical method can produce extremely high sensitivity for antibiotic analysis and devote to study the toxicity to human beings.

Experimental

Experimental Scheme. Figure 1 illustrated the experimental scheme to determine salinomycin using ICP-MS. Since the size of salinomycin was too small to have a sandwich-type immunoreaction, it was designed to directly react with amine groups functionalized on magnetic nanoparticles (MNPs) by forming an amide bond for magnetic extraction. Then, antibody-immobilized TiO_2 nanoparticles in the size of ~10 nm was selectively tagged on the salinomycin target through antigen-antibody reaction. The concentration of salinomycin was determined quantitatively by the measurement of ^{49}Ti signal using ICP-MS.

Synthesis and Functionalization of Nanoparticles. The surface-modified Fe_3O_4 MNPs with amine-functional group were synthesized by a co-precipitation method for magnetic

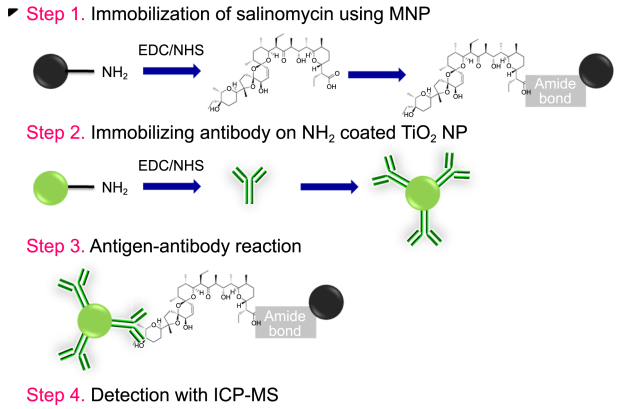

Figure 1. Scheme of the experimental procedure for the detection of salinomycin using magnetic and TiO_2 particle tagging.

cores, followed by the formation of silica shell with TEOS (Tetraethyl orthosilicate 99.999%, Sigma-Aldrich Chem. Co., USA) and the functionalization of amine group with APTEOS (3-Aminopropyl triethoxysiliane 99%, Sigma-Aldrich Chem. Co., USA).[17-19] In brief, the superparamagnetic Fe_3O_4 MNPs were synthesized through the alkaline co-precipitation of $FeCl_2 \cdot 4H_2O$ (0.5 g) and $FeCl_3 \cdot 6H_2O$ (1.35 g) dissolved in 25 mL of de-ionized water in an Ar gas flow. Then, 12.5 mL of ammonium hydroxide (28-30%, Sigma-Aldrich Chem. Co., USA) was added and heated to 80 °C for 20 min. For the silica shell, 200 μL TEOS was added into 100 mL of ethanol (80%) containing 100 mg of MNPs in the presence of ammonium hydroxide at pH 9. Then amine-functionalized SiO_2 layer was formed by the addition of 1 mL of APTEOS and reacted for 24 h. The nanoparticles were washed with acetone three times and stored in ethanol for the next step. TiO_2 nanoparticles in the size of ~10 nm were synthesized by a sol-gel method using titanium isopropoxide (TTIP, 97%, Sigma-Aldrich Chem. Co., USA) and ammonium hydroxide in ethanol. For the reaction, a mixture of ethanol, TTIP, and ammonium hydroxide in the volume ratio of 3:2:1 was reacted under vigorous stirring. Powder of TiO_2 NPs can be obtained by solvent evaporation. For silica coating, a mixture of ethanol, water, and TEOS in the volume ration of 50:20:1 was added drop-wisely after the addition of ammonium hydroxide for pH 9.

Extraction and Measurement. For the measurement of salinomycin in serum (Sigma-Aldrich Chem. Co., USA), 5 g of sample was dispersed in 10 mL of hot water and spiked with salinomycin for standard addition. For the elimination of proteins and fats, 20 mL of acetonitrile (CH_3CN) was added and then the precipitates were separated by centrifuging. After the filtrate was treated with 8 mL of hexane (C_6H_{14}) and sonicated, the upper solution was collected using a PTFE syringe filter with a pore size of 0.2 μm for the next steps. For the extraction, the salinomycin was bonded to the MNPs after the carboxylic group was activated by the mixture (2:1) of 1-ethyl-3(dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), as shown in Figure 1. The unreacted materials were washed out five times by magnetic separation. Polyclonal IgG (antibody of salinomycin) labelled with the synthesized TiO_2 nanoparticles reacted with the salinomycin for 30 min through immunoreaction and the final product was separated by centrifugation. All the washing and reactions for the amide bond and the immunoreaction were carried out in phosphate buffered saline (PBS) solutions. The ^{49}Ti signal of the reaction product were measured by ICP-MS (DRC-e, Perkin-Elmer, USA) equipped with a flow injection system (Rheodyne, Analytical Injector 9725, USA) of 10 μL injection loop and a PFA nebulizer (Gemclean crossflow II nebulizer, Perkin-Elmer, USA) with a Ryton Scott-type spray chamber. The operating condition for ICP-MS is listed in the supplementary table. The amine groups functionalized on the NPs were identified by a lab-built laser induced fluorescence microscopy (LIFM) equipped with a CCD camera (Micro Publisher 5.0, Q-Imaging) and a 473 nm DPSS Laser (50 mW, BL473T-050, SLOC) for excitation after tagging FITC. FT-IR (Spectrun100, PerkinElmer Co.) was also used for the identification of functional groups modified on the nanoparticles. Images of the synthesized NPs were obtained by a transmission electron microscope (JEM1010, JEOL, Japan).

Results and Discussion

Selection and Optimization of Reactions. The core sizes of Fe_3O_4 MNPs and TiO_2 NPs synthesized and modified in this work were 10.1 nm (± 3.1) and 11.2 nm (± 2.5), respectively (supplementary figure 1). Since both NPs were small enough, they exhibited excellent particle activity in various reactions of the treatment procedure shown in Figure 1. In addition, the sizes of NPs are small enough to be completely dissociated in ICP, which can result in high ionization efficiency and good reproducibility in ICP-MS measurement. However, these nanoparticles were not small enough for the sandwich-type extraction model due to the geometrical problem in order to determine antibiotics like salinomycin, which is generally much smaller than biomarkers. We therefore employed a non-specific extraction model (Fig. 1) for the target collection and probing, in which the small antibiotics were directly bound to the MNPs without antibodies and then tagged by probe nanoparticles through the immunoreaction. Since both nanoparticles, MNPs and TiO_2 NPs, were synthesized in the diameter of ~10 nm, the well-balanced dumbbell-type final products can be formed because they were dispersed well and evenly reacted owing to their excellent activity. Generally, the size of TiO_2 NPs was more important than that of MNPs in this case because MNPs have a limitation in size in order to keep the superparamagnetic property.

The reaction conditions of each step were optimized for maximum measurement sensitivity and stability of the developed method.[20] For example, Figure 2 illustrated the intensity changes of ^{49}Ti in ICP-MS when the reaction time was increased. Noticeably, the maximum intensity reached at 60 min for the reaction of salinomycin binding to MNPs (Fig. 2(a)) when a microwave stirring was used. The reaction time of the microwave was much shorter than that of a

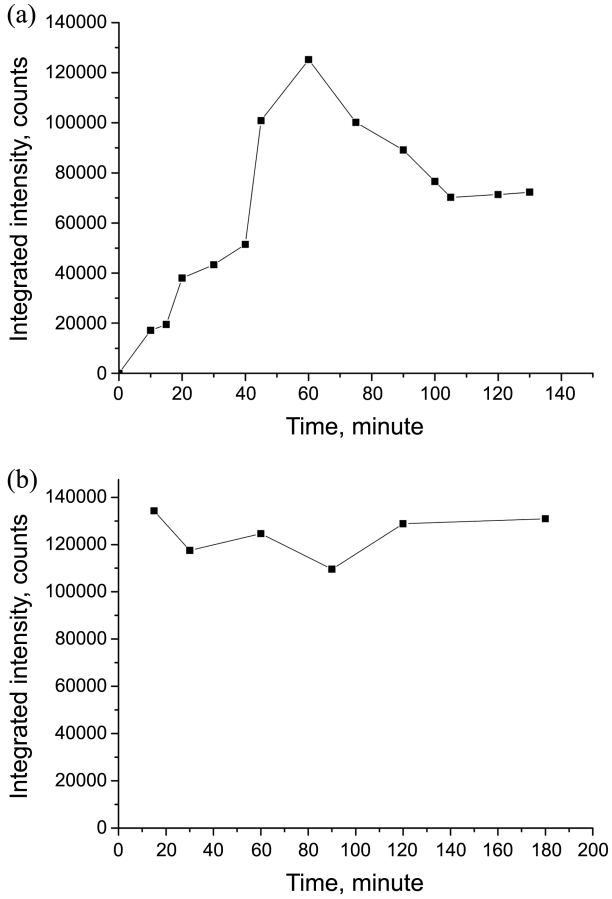

Figure 2. Optimization of reaction time for the binding of salinomycin to MNPs (a) and for the TiO_2 tagging (b).

mechanical stirring, which generally required almost a day for completion. However, the use of microwave can change the reaction condition because of heat generation, which probably resulted in maximized reaction efficiency at 60 min. The signal intensity for the TiO_2 tagging through antigen-antibody reaction was almost unchanged if it passed over 20 min (Fig. 2(b)). Therefore, the extraction and tagging reactions were optimized to be 60 min and 20 min, respectively. Experimentally, these two procedures were the most time-consuming steps to determine salinomycin in this model.

Measurement of Salinomycin. Although the magnetic extraction provided a good speed and an excellent efficiency, it showed relatively high background level because of non-specifically adsorbed molecules. Therefore, the MNPs might be added excessively in order to collect the target completely if possible.[21] In addition, the interfering molecules of the sample matrix should be removed as perfectly as possible during the sample pretreatment procedure. Fortunately, since the matrix of serum was not so heavy as fresh-meat samples, the background level was not so high in the method described in the experimental section. Furthermore, blank subtraction using a verified serum sample and standard addition were carried out in this work.

For quantification, the concentration of antibiotic target

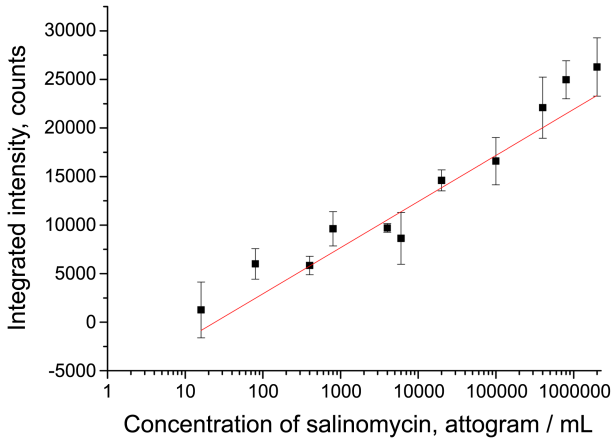

Figure 3. Experimental plot of the ^{49}Ti intensity measured by ICP-MS *vs* the concentration of salinomycin (3 repeated measurements).

spiked to the serum should be proportional to the signal intensity of TiO_2 nanoparticles measured by ICP-MS. Figure 3 illustrated the plot of ^{49}Ti intensity *vs* the spiked concentration of salinomycin. Although those data points were obtained at three different experimental sets, the integrated ^{49}Ti signal showed almost the linear response to the logarithm of concentration change over the remarkably long linear dynamic range, *i.e.*, about 16 to over 10^6 ag mL^{-1}. From the curves, the averaged detection limit of 0.4 ag mL^{-1} was obtained (3 σ/m, where σ (= 709.7 counts) was the standard deviation of blank and m was the slope), which corresponded to about 310 salinomycin molecules when 10 μL injection was used. The obtained numbers of salinomycin molecules were in the reasonable range because total number of Ti atom in a nanoparticle was estimated to be about 4,000 atoms by considering the particle size and density and the throughput of ICP-MS. The result was more than 1.5×10^6 times lower than that of LC-MS/MS reported in the reported articles[6] and about 1.2×10^{11} times better than that of ELISA.[17] At this moment, a ELISA kit (Kwinbon biotech, China) was used for verification and the measurement procedure was followed by the manufacturer's instruction manual. Such a remarkable sensitivity and analytical performance achieved in this method maybe come from a significant signal amplification by the large number of atoms per a target and the excellent detection capability of ICP-MS. Furthermore, ICP-MS also contributed to the extraordinarily long linear dynamic range in calibration.

Cross Reactivity. Since the extraction procedure was non-specific, cross reactivity was studied for the antibiotics containing the same carboxylic group, and the results were shown in Figure 4. Ciprofloxacin showed about 20% signal interference to the salinomycin and the other antibiotics like enrofloxacin and sulfathizole exhibited slightly lower level of interferences. Since the cross reactivity was inherently affected by the immunoreaction, no further effort to improve the method selectivity can be made in this work except the matrix cleaning.

In conclusion, the developed detection method to deter-

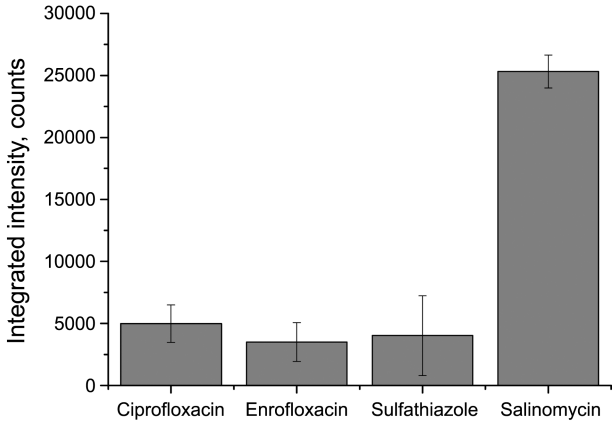

Figure 4. Cross reactivity of salinomycin for ciprofloxacin, enrofloxacin and sulfathiazole in ICP-MS with TiO_2 particle tagging.

mine salinomycin in serum showed excellent analytical performances. The linear dynamic range increased more than 10^5 times and the detection limit was improved by the factor of 10^4 to 10^9, compared to the typical analytical methods, like LC-MS/MS and ELISA. These excellent performances resulted from the signal amplification of nanoparticles tagged on the target incorporated with the excellent detection capability of ICP-MS.

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