# AUTOMATIC ELISA ANALYTICAL SYSTEM FOR A TRACE AMOUNT OF ENVIRONMENTAL CHEMICALS USING A 3-DIMENSIONAL MICROREACTOR WITH A NOVEL ANTIGEN-BOUND MICROFILTER

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## ABSTRACT

We developed an automatic analytical system for the determination of an endocrine disrupting chemical, nonylphenol (NP), which employs a competitive Enzyme-Linked ImmunoSorbent Assay (ELISA) using a novel antigen-bound microfilter. The filter was set in a unique 3-dimensional microreactor, and all the fluidic processes for the reaction and detection were automatically performed according to a PC program in the system. By using this system, we obtained a negative standard curve, of which NP concentration range (0.01-0.40 μg/L) was much lower than the quantitative limit (5 μg/L) of a conventional ELISA kit for NP measurement. The coefficients of the variation were comparable to those of commercial products.

## KEYWORDS

ELISA, antigen-bound microfilter, microreactor, nonylphenol, automatic analytical system

## INTRODUCTION

Since 1970s, Enzyme-Linked ImmunoSorbent Assay (ELISA) using specific reactions between antigen and antibody has been developed and used mainly in biomedical fields to detect peptides, hormones, antibodies, pathogenic microbes, and viruses owing to its convenience, specificity, and good sensitivity [1]. The assay generally uses 96-well microtiter plates, a plate washer, and a plate reader, and it takes 2 h or more to get results, although it is well automated. The plate reader and washer occupy a certain area (typically at least 1 m x 0.6 m) on the table and are equipped in specific analytical rooms in hospitals, institutes, universities, and analytical companies. Therefore, samples to be analyzed must be sent to such specific facilities, resulting in long-time waiting and high costs. However, recently, more rapid on-site analysis is required in various cases. For instance, in food markets, residual chemicals in food and pathogenic bacteria should be frequently checked on site and at real time for the safety and rapid sale of fresh food [2,3]. Thus, development of portable automatic analytical systems with high sensitivity is desired.

In this study, we established an automatic ELISA analytical system for the determination of nonylphenol (NP), which is one of the representative endocrine disrupting chemicals and was selected as a model low molecular weight compound. Three novelties were integrated into this system: (i) a competitive ELISA using a newly designed and prepared antigen-bound microfilter, (ii) a unique 3-dimesional microreactor with two microfilters for reaction and mixing, and (iii) an automatic fluidic and detection system consisting of five pumps, an LED light source, a palm-top spectrophotometer, a micro-flowcell, and a lap-top PC. The use of the microreactor can reduce the volumes of reagents to be used due to its smaller size. In addition, the use of the microfilter can reduce the reaction times due to its high reaction efficiency based on the huge reaction area. The automatic system can prevent errors by manual handling and improve the data reliability.

As the first step for the development of the future portable system, we here prove the principle of this competitive ELISA and the usefulness of this system for determination of a trace amount of low-molecular weight chemicals.

## EXPERIMENT

For the antigen-bound microfilter, we newly developed a polymethylmethacrylate (PMMA)-based polymer, into which nonylphenyl group was incorporated at 5% in molar ratio instead of the methyl group of PMMA (Fig.1). The incorporation of the nonylphenyl group was confirmed by $^1$H-NMR analysis. The film of the polymer was prepared using a nanoimprint machine ST-50 (Toshiba Machine, Numazu) and a pair of Teflon plates (10 cmϕ) with a 200-μm Teflon spacer. The microfilter (3mmϕ x 200μm) was prepared from the polymer film by synchrotron irradiation using New SUBARU BL-2 (Hyogo pref., Japan) as described previously [4] and had more than 2100 40-μm bores in the 3-mmϕ area. It was set in a unique 3-dimensional microreactor (24 mmϕ x 26 mm), which had a 3-mmϕ vertical path and therefore enables vertical fluidic operation (Fig.1). Another cross-linked capillary type filter [5] was also set in the reactor for mixing as shown in Fig.1.

For the new competitive ELISA, samples including various concentrations of NP were mixed and reacted with anti-NP monoclonal antibody (AP-159, Japan EnviroChemicals, Osaka) conjugated with horseradish peroxidase (HRP) in small test tubes (Fig.2). The conjugate was prepared using an HRP labeling kit (Cosmo Bio, Tokyo) according to the instruction. Then, the mixture was introduced into the microfilter in the microreactor, where only antibody unsaturated with NP at its paratopes can bind to the nonylphenyl group exposed on the surface of the filter



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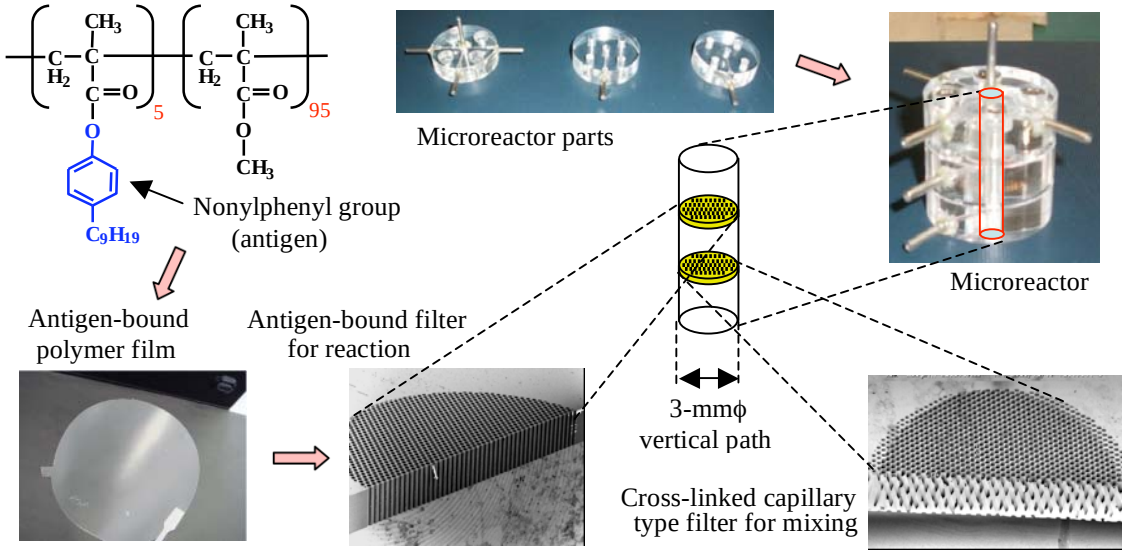



*Figure 1. Antigen-bound microfilter and microreactor*

bores (Fig.2). After washing the path and the filters with washing buffer (10 mM sodium phosphate buffer including 0.05% (v/v) Tween20, pH7.0), a fluorescent substrate for HRP, 3-(4-hydroxyphenyl)propionic acid (HPPA), and $H_2O_2$ were introduced into the filter to develop fluorescence (420 nm) depending on the activity of the HRP trapped on the surface of the bores. When NP concentration in the sample is low, most antibodies remain unsaturated in the tube, giving high fluorescent count. In contrast, when the NP concentration is high, most antibodies react with NP in the tube and thus can't be trapped with the nonylphenyl group on the surface of the filter bores (Ftg.2), resulting in low fluorescent count. Finally, this competitive ELISA gives a negative standard curve.

The microreactor was set in an automatic fluidic and detection system consisting of 5 PC-controlled pumps (NE-1000 x 4, NE-9000 x 1, New Era, NY), a high power LED light source (325 nm) (LLS-325, Ocean Optics, FL), a micro-flowcell (FIA-SMA-FL, Ocean Optics), a palm-top spectrophotometer SEC2000-UV/VIS (BAS, Tokyo), and a lap-top Windows PC (Fig.3). The pumps work according to a program running on WIN PUMP TERM (New Era). The program for the fluidic operation was as follows: injection of 200 μl of the reaction mixture in 8 s (Pump2); kept for 25 min at the microfilter (for the reaction of the unsaturated antibodies with the nonylphenyl group exposed on the surface of filter bores); washing with 2.7 ml of the washing buffer in 24 s (Pump1); flushing the path and the filter with 150 ml of air in 30 s (Pump5); injection of 200 μl of fluorescent substrate solution (HPPA + $H_2O_2$) in 8 s (Pump3); kept for 15 min at the microfilter (for the reaction of the HRP with the fluorescent substrate); injection of 2.7 ml of 0.1 M glycine-NaOH solution (pH10.3) (termination solution) in 48 s (Pump4); washing for the next analysis with 2.7 ml of the washing buffer in 48 s (Pump1). Total analytical time was about 50 min. The reactor was kept at 37 °C in an incubator during this operation.

At first, we prepared standard NP solutions (0.1-3.2 μg/L) in 5% MeOH and mixed them (100 μl each) with the same volume of anti-NP antibody-HRP conjugate solution

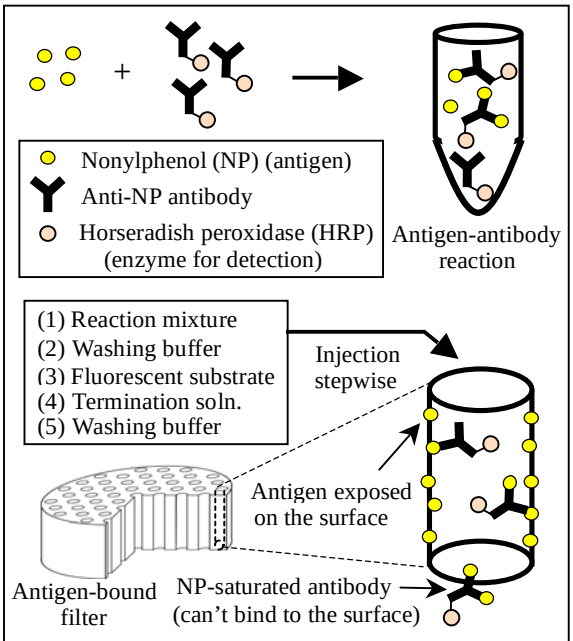
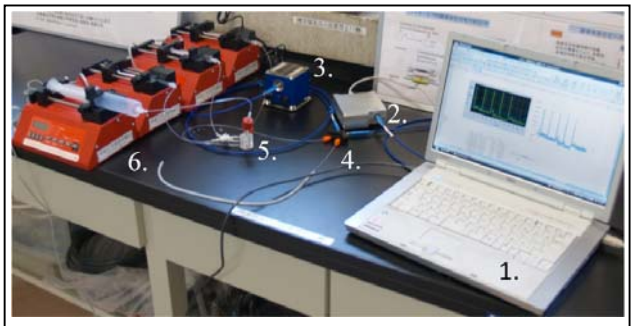



*Figure 2. Competitive ELISA using antigen-bound microfilter*

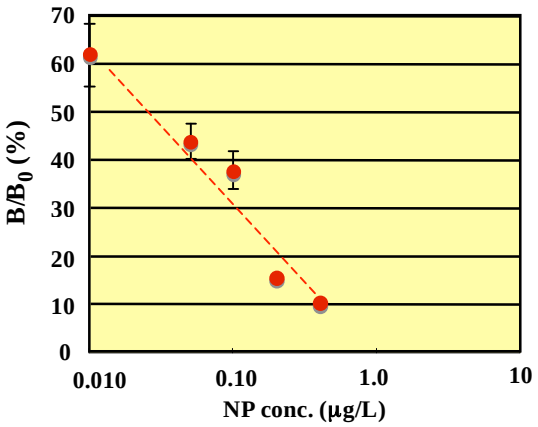



1. PC for control and data recording, 2. Palm-top spectro-photometer, 3. High power LED light source, 4. Micro-flowcell, 5. Microreactor, 6. PC-controlled pumps.

*Figure 3. Overview of automatic ELISA analytical system.*

(approx. 0.32 μg/mL). The mixture was kept for 1 h at 37 °C for antigen-antibody reaction. Then, using the mixtures, we measured their NP concentrations by manual handling (not using the automatic system). As a result, a good negative standard curve was obtained at the concentration range, giving 40% to 90% of the $B/B_0$ values, where B is the fluorescent count at the concentration and $B_0$ is the fluorescent count at 0 μg/L. This result indicates that this competitive ELISA can apply for the measurement of a trace amount of low molecular weight chemicals. However, the coefficients of the variation (CVs) were over 15% (15-40%) and its repeatability was also bad. Errors might be caused by the manual handling of small volumes and time lags during fluorescent measurements (in the manual handling, the effluent from the reactor was once sampled and its fluorescence was measured using a fluorescence spectrometer FP-6300 (JASCO, Tokyo)).

To reduce such errors, we set the microreactor in the automatic fluidic and detection system and measured standard NP solutions again. However, in the first version of the automatic system using five NE-1000 syringe pumps, we frequently observed air bubbles in the vertical path during the operation and they affected the stopping positions of the solutions at the microfilter. Therefore, the CVs were very bad (18-49%). We found that the occurrence of the bubbles resulted from the wetness of the path surface. Thus, we replaced one NE-1000 pump for air flushing into a high flow rate peristaltic pump NE-9000, together with some other modifications. Finally, using the revised system, by 5 determinations each at 6 concentration points, we obtained a negative standard curve between 0.01 μg/L and 0.40 μg/L (Fig.4). The CVs were significantly improved (9.1-12.4%), which were comparable to those of commercial products (<10%). Recent further improvement achieved approx. 5 % of the CVs (data not shown). In addition, this concentration range was 10 times lower than the quantitative limit of a conventional ELISA kit for NP measurement (5 μg/L, Japan EnviroChemicals) and below the predicted no effect concentration of NP for fish (0.608 μg/L) [6]. Since we have not optimized the analytical conditions yet, it is possible to make better standard curves and shorten the reaction times using this automatic ELISA analytical system.




B: Fluorescent count at each concentration.
$B_0$: Fluorescent count at 0 μg/L.

*Figure 4. Standard curve in the measurement of NP using the automatic ELISA analytical system.*

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