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Fully automated immunoassay system of endocrine disrupting chemicals using monoclonal antibodies chemically conjugated to bacterial magnetic particles

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Abstract

The development of a rapid and high-throughput detection system for endocrine disrupting chemicals (EDCs) has been required in the recent years. A fully automated immunoassay system was described for the detection of EDCs, such as alkylphenol ethoxylates (APEs), bisphenol A (BPA) and linear alkylbenzene sulfonates (LASs), using monoclonal antibodies chemically conjugated to bacterial magnetic particles (BMPs) and alkaline phosphatase (ALP)-conjugated EDCs. EDC concentrations were evaluated by the decrease in luminescence based on the competitive reaction of EDCs and ALP-conjugated EDCs. Full automation of the BMP-based immunoassay was achieved by using an automated eight-way pipet moving at *X*-, *Y*- and *Z*-direction and a *B*/*F* separation unit. *B*/*F* separation was performed on the tip surface of eight-way pipet with a retractable magnet mounted close to the pipet tip. Immunoreactions were saturated after 10 min, and the assay was completed within 15 min. The detection ranges for APE, BPA and LAS were 6.6 ppb–66 ppm, 2.3 ppt–2.3 ppm, and 35 ppt–35 ppm, respectively. This BMP-based immunoassay system has advantages due to its high sensitivity and rapid measurement of samples.

Keywords: Bacterial magnetic particles (BMPs); Endocrine disrupting chemicals (EDCs); Fully automated immunoassay system; Alkylphenol ethoxylates (APEs); Bisphenol A (BPA); Linear alkylbenzene sulfonates (LASs)

1. Introduction

Over the last few years, a variety of environmental contaminants have been reported to adversely affect humans and wildlife through interactions with the endocrine system. These compounds have been broadly defined as environmental endocrine disrupting chemicals (EDCs). EDCs are routinely measured by gas chromatography–mass spectrometry (GC–MS) or high performance liquid chromatography (HPLC) [1–3]. GC–MS and HPLC are highly sensitive and specific, but cannot process multiple samples rapidly.

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Furthermore, the preparation of samples for gradient separation and purification is time consuming. Therefore, the development of a rapid and high-throughput detection system for endocrine disrupters has been undertaken.

In this study, alkylphenol ethoxylates (APEs), bisphenol A (BPA) and linear alkylbenzene sulfonates (LASs) were evaluated. Alkylphenol ethoxylates (APEs) are routinely used in pesticide formulations. Biodegradation of APE results in the accumulation of persistent short chain mono-, di- and tri-ethoxylates which are more toxic than the parent compounds and potentially estrogenic [4]. APE degradation products such as nonylphenol and octylphenol are shown to have estrogenic effects [5,6]. Bisphenol A (BPA) is a widely used monomer in the manufacture of polycarbonate plastics and epoxy resins and has been reported to have estrogenic effects [3,7]. Linear alkylbenzene sulfonates (LASs) are major ingredients in synthetic detergents and surfactants and used worldwide for both domestic and industrial applications [8]. LAS are toxic and regulated environmentally at 0.2 mg/l as 'toxic' level, not estrogen-affected level.

The detection of alkylphenol, BPA and LAS using enzyme-linked immunosorbent assay (ELISA) has been reported [9,10]. In general, the microparticle-based immunoassay enables us to detect rapidly the antigens with high sensitivity as compared with the microtiter plate-based immunoassay because of the increase in the reaction surfaces.

Magnetic bacteria isolated from freshwater and marine sediments are known to synthesize magnetite particles [11–14]. Bacterial magnetic particles (BMPs) are 50–100 nm in size and disperse in solution well because of their stable lipid membrane [15]. Highly sensitive detection has been successfully achieved by using BMPs as carriers for functional molecules, such as enzyme [16], antibodies [15,17] and DNA [18,19]. Furthermore, a fully automated immunoassay system [20] and high-throughput DNA detection system [21] using BMPs have been developed.

In this paper, we developed a sensitive and rapid automated detection system for EDCs including APE, BPA and LAS using antibodies chemically conjugated to BMPs. The cross-reactivity (%) of antibodies against EDCs was also evaluated.

2. Materials and methods

2.1. Materials

Nonylphenol ethoxylates (ethoxylate region: 2–20; APE) as alkylphenol ethoxylates and linear alkylbenzene sulfonates (LASs) were obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Nonylphenol, bisphenol A (BPA), sodium dodecyl sulfate, 4,4′-dihydroxy diphenyl and 1-ethyl-3-(3-dimethylaminopropyl) carobodiimide hydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). The 17β-estradiol and *N*-hydroxy succinimide (NHS) were obtained from Sigma Chemical Co. (MO, USA). The 3-aminopropyltriethoxysilane (APTES) was obtained from Shin-Etsu Chemical Co. Ltd. (Tokyo, Japan). Bis(sulfosuccinimidyl)suberate (BS3) was purchased from Pierce Chemical Co. (Rockford, IL, USA). Alkaline phosphatase (ALP) was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Lumi-Phos 530, which includes lumigen PPD, 4-methoxy-4-[3-(phosphonoxy)phenyl] spiro[1,2-dioxetane-3,2′-adamantane] disodium salt (3.3×10^{-4} M), was obtained from Wako Pure Chemical Industries. Artificial magnetic particles (AMPs) consisting of magnetite (approximately 250 nm in diameter) were kindly gifted from TDK Corp. (Tokyo, Japan). Deionized distilled water was used in all procedures.

2.2. Preparation of BMPs

Magnetic bacterium, *Magnetospirillum magneticum* AMB-1, was grown in modified magnetospirillum growth medium (MSGM) [22] at room temperature, under microaerobic conditions for 7–10 days. Bacteria were grown to stationary phase and centrifuged at 10,000 × *g* for 10 min at 4 °C. The collected cells were suspended in phosphate buffered saline (10 mM PBS, pH 7.4), and disrupted by three passes through a French press cell at 1500 kg/cm^2 (Ohtake Works Co. Ltd., Tokyo, Japan). BMPs were collected from disrupted cell fractions using a columnar neodymium-boron (Nd-B) magnet (diameter 22.5 nm, height 12.5 nm) that produced an inhomogeneous magnetic field (0.5 T at the surface). Collected BMPs were washed with 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic

acid (HEPES) buffer (10 mM, pH 7.4) using an ultrasonic bath at least three times, and stored at 4 °C in 10 mM PBS.

2.3. Chemical conjugation of anti-EDCs antibodies on BMPs (Ab-BMPs) and AMPs (Ab-AMPs)

BMPs (1 mg) and AMPs (1 mg) were suspended in 1 ml of 1 mM BS3 and incubated for 30 min at room temperature with pulsed sonication (1 min pulses at 5 min intervals). AMPs were pre-treated with APTES to introduce amino residues on their surface. BS3-modified BMPs and AMPs were separated magnetically from the reaction mixture using an Nd-B magnet and washed three times with 1.0 ml of 10 mM PBS. Modified BMPs and AMPs were incubated with 100 μg/ml of monoclonal antibody at room temperature for 1 h with pulsed sonication (1 min pulses at 5 min intervals). Anti-APE, BPA and LAS monoclonal antibodies (IgG) were prepared as previously described [9,10]. Excess antibody was removed from antibody chemically conjugated to BMPs (Ab-BMPs) and AMPs (Ab-AMPs) by washing three times with PBS.

The size distribution and average diameters were evaluated using a laser particle analyzer (PAR-III, Otsuka Electronics, Osaka, Japan). BMPs in the 40–200 nm size range, or AMPs in the 40–480 nm size range were defined as monodisperse particles.

2.4. Preparation of alkaline phosphatase conjugated to EDCs (ALP-EDCs)

Each EDCs was carboxylated [9,10], and subsequently esterified using *N*-hydroxy succinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carobodiimide hydrochloride (ECDI). The succinimide ester of EDCs was reacted with the amino residue of ALP to conjugate each other. Carboxylated APE was synthesized as following: poly(ethyleneglycol) monononylphenylether ($n = 5$, 11.4 mmol) was dissolved in 60 ml toluene. Absolute succinic acid (9.2 mmol) and *p*-toluene sulfonate (0.08 mmol) was added to the solution, and agitated at 80 °C for 2 h. After cooling, the mixture was evaporated to remove toluene. A 5% potassium carbonate solution was added to the remaining solution to adjust to pH 12. After washing the solution with ether, the aqueous fraction was adjusted to less than pH 2 by the addition of hydrochloric acid. The product was extracted by chloroform. The chloroform fraction was washed with saturated NaCl solution, and evaporated to dryness to obtain the final products. Carboxylated BPA was synthesized as following: BPA (25 g) and glutaric acid (12.5 g) were reacted at 100 °C for 18 h under N_2 atmosphere. After cooling, transparent and oily fraction was dissolved in ethyl acetate, and extracted using silica gel column. A content adsorbed on the column was eluted with hexane–ethyl acetate (1:1), and concentrated. The solid (4.45 g) was dissolved in 50 ml methanol, applied to ODS column (3 cm × 50 cm) and eluted with 70% ethanol. After separation by TLC (RP-18), an oily product was extracted with ethyl acetate, and concentrated. The purified fraction was dissolved in isopropanol and crystallized with hexane. The final product was washed with hexane, and evaporated to dryness. Carboxylated LAS was prepared as described previously [9].

Each carboxylated EDC (0.1 mmol) was dissolved in 1 ml dimethylsulfoxide (DMSO). The solution was added to 1 ml DMSO containing 0.14 mol of NHS and 0.14 mmol ECDI for APE and LAS, and 0.18 mmol of NHS and 0.52 mmol of ECDI for LAS. The mixture was agitated overnight at room temperature. The NHS-modified EDCs (6 μl) were mixed with 5 mg ALP in 5 ml PBS and incubated overnight at 4 °C. After incubation, excess reagents were removed by Centricon YM-30 (Millipore Corp., MA, USA). The ALP conjugated to EDCs (ALP-EDCs) were diluted 1000-fold and used. The final ALP-EDCs concentration was approximately 5 μg/ml.

2.5. Cross-reactivity of anti-APE, BPA and LAS antibodies

To evaluate the cross-reactivity of antibodies against 10 μM analogue compounds, artificial magnetic particles were used. Luminescence intensity (LI) was measured when an antigen was used in competitive immunoassay. Cross-reactivity was determined through the equation:

$$\text{cross-reactivity} = \frac{\text{LI(zero dose)} - \text{LI(analog compound)}}{\text{LI(zero dose)} - \text{LI(antigen)}} \times 100$$

LI (zero dose) shows the luminescence intensity obtained when only ALP-EDCs were used in competitive immunoassay.

2.6. Fully automated immunoassay of EDCs using Ab-BMPs

A fully automated immunoassay system [20] was used for all assaying procedures of EDCs including APE, BPA, and LAS, see Fig. 1. The system was comprised of a reaction station, a tip rack (8 × 3 tips for reaction), and an automated eight-way pipet bearing a retractable magnet mounted close to the pipet tip corresponding to the 96-well microtiter plate. The microtiter plate was mounted in the reaction station. There is one rack holding 8 × 3 tips for reaction. Specially designed polypropylene tips were used for this experiment. A thin tip end and intermediate section in the tip was used as a reservoir to aspirate the reaction mixture. At the intermediate section, magnetic separation was performed by loading the tip surface with magnet during aspiration or dispensing of suspensions of Ab-BMPs. Resuspension of trapped BMPs on the inner surface of the tip was performed by aspirating and dispensing PBS without a magnet. As shown in Fig. 1, the immunoassay involved the separation of the immunocomplexes containing anti-EDCs Ab-BMPs from suspensions at 7 different stages: (1 and 2) separation of Ab-BMPs following immunoreaction; (3–5) three washes of the immunoreaction product; (6 and 7) ALP enzyme reaction and measurement of luminescence intensity. ALP-antigen

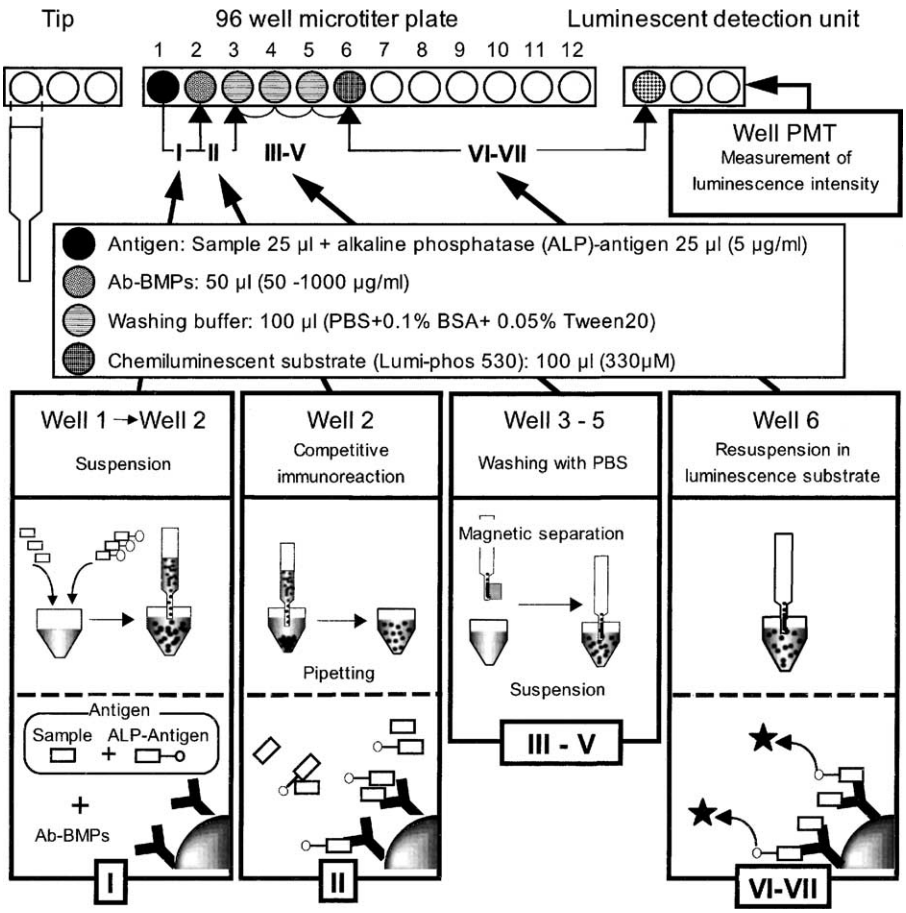

Fig. 1. Experimental procedure for competitive immunoassay using a fully automated system.

(ALP-EDCs, 25 μl) and sample solution containing EDCs as antigen (25 μl) were mixed with a suspension of Ab-BMPs (50 μl). The mixture was sonicated in the ultrasonic bath for 10 min (10 s pulses at 50 s intervals) at room temperature. Antigen and Ab-BMPs were magnetically separated from the mixture using an Nd-B magnet and then washed three times by resuspension in 100 μl of PBS containing 0.1% (w/v) BSA and 0.05% (v/v) Tween-20 (20 cycles of pipet action). Finally, the complexes were suspended in 100 μl of luminescent substrate, Lumi-Phos 530, incubated for 3 min, and the luminescence intensity was determined.

2.7. Precision of the fully automated immunoassay system

The precision of the competitive immunoassay method was evaluated by within-day and between-day coefficients of variation (CV). The within-day CV and between-day CV were expressed as intraassay CV and interassay CV, respectively. Samples of 1 nM EDCs were assayed in triplicates to determine precision intraassay, and assayed three times in multiple assays to determine precision interassay. The assay for APE, BPA and LAS was performed simultaneously using the fully automated system. For simultaneous determination of EDCs, each antigen was deposited into different wells.

2.8. ELISA of EDCs

Flat-bottom polystyrene microtiter plates (Coaster Co., NY, USA) were coated with 100 μl per well of antibody (10 μg/ml) in PBS, and incubated for 30 min at room temperature. After washing three times with washing buffer (PBS containing 0.1% (w/v) BSA and 0.05% (v/v) Tween-20), PBS containing BSA (100 μg/ml) was added to plates to prevent non-specific adsorption, and incubated for 30 min at room temperature. The mixture containing 25 μl of 5 μg/ml ALP-antigen and 25 μl of the sample was added to the antibody-conjugated wells, and incubated for 1 h at room temperature. After washing three times, 100 μl of Lumi-Phos 530 (100 μM) was added to the plates, and incubated for 5 min at room temperature. Luminescence intensity was measured using a luminometer (Lucy-2, Aloka, Tokyo, Japan).

3. Results and discussion

3.1. Conjugation of anti-EDCs antibodies on BMPs

The conjugation of anti-EDCs antibody onto BMPs was done through the use of a homofunctional cross-linker, BS3. BS3 has two sulfosuccinimidyl residues which are reactive to an amino residue. Reaction was completed within 1 h, which is much more rapid than previous methods using heterofunctional cross-linkers, Sulfo-LC-SPDP and Sulfo-SMCC [23]. The heterofunctional cross-linker has two different residues reactive to an amino and thiol residues, respectively. Moreover, the amounts of Ab-BMPs increased by about 1.7-fold to 94 μg/mg (anti-BPA antibody) of BMPs. No significant difference was observed in amounts of antibody chemically conjugated to BMPs when using anti-APE, anti-BPA and anti-LAS antibodies. The amount of antibodies reached approximately 10% (w/w) of BMPs. BS3 is a better cross-linker reagent than Sulfo-LC-SPDP and Sulfo-SMCC for the conjugation of antibodies onto BMPs.

3.2. Immunoreactions using Ab-BMPs

Fig. 2 shows the time course of immunoreaction using anti-BPA Ab-BMPs. The luminescence

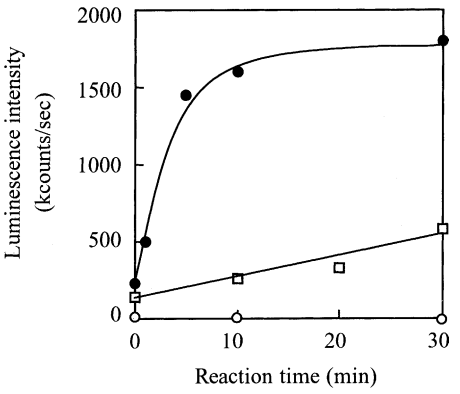

Fig. 2. Time course of luminescence intensity when using anti-BPA Ab-BMPs (closed circles), non-treated BMPs (open circles) and anti-BPA antibody-immobilized microtiter plate (open squares). Ab-BMPs concentration, ALP–BPA concentration and luminescence substrate concentration were 500, 5 μg/ml and 330 μM, respectively. The total reaction volume was 100 μl.

intensity during the immunoreaction increased rapidly in 10 min, and became almost constant after 10 min. The immunoreaction using anti-APE and anti-LAS antibodies was also done within 10 min. The washing steps, alkaline phosphatase reaction, and measurement of luminescence intensity were accomplished within 5 min. All measurement steps were completed in 15 min. In contrast, when anti-BPA antibodies immobilized on microtiter plates were used, the reaction time of at least 1 h was required. All measurement steps took more than 2.5 h. Furthermore, higher luminescence signals were obtained with the BMP system than that with the ELISA system, indicating high sensitivity of the BMP system.

To optimize Ab-BMPs concentration, the relationship between Ab-BMPs concentration and luminescence intensity was evaluated. Fig. 3 shows that luminescence intensity increased with increasing anti-BPA Ab-BMPs concentrations. Luminescence intensity reached a plateau at over 500 μg/ml of Ab-BMPs. This result suggests that the binding sites of antibodies to antigens on BMPs were saturated at over 500 μg/ml, or the binding sites were not saturated but the reaction surface on BMPs was saturated because of the aggregation of BMPs at over 500 μg/ml. The similar

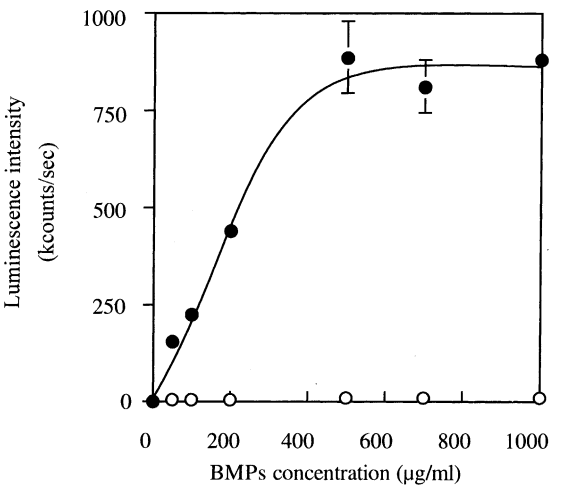

Fig. 3. Relationship between luminescence intensity and Ab-BMPs concentration. ALP–BPA concentration and luminescence substrate concentration were 5 μg/ml and 330 μM, respectively. Antigen–antibody reaction time was 10 min. The total reaction volume was 100 μl. Closed circle shows the luminescence intensity when using anti-BPA Ab-BMPs. Open circle shows the luminescence intensity when using non-treated BMPs.

tendencies were observed when using anti-APE and anti-LAS Ab-BMPs. The increased luminescence intensity was dependent on the antigen–antibody reaction, not the non-specific adsorption of ALP-antigen onto BMP surfaces since no luminescence was detected when using unmodified BMPs.

To evaluate the precision of fully automated immunoassay system, CVs in simultaneous determination of APE, BPA and LAS were calculated. The intraassay CVs using anti-APE, anti-BPA and anti-LAS Ab-BMPs were 7.0, 4.1, 7.4%, respectively. The assay using anti-BPA Ab-BMPs was more precise than that using other antigens. The interassay CV using anti-BPA Ab-BMPs was 10.4%. On the other hand, the CVs of intraassay and interassay using anti-BPA Ab-BMPs were 7.4 and 10.4%, respectively, when the measurements were done manually. This suggests that the fully automated system with Ab-BMPs was precise.

3.3. Competitive immunoassay for APE, BPA, and LAS

Detection ranges of APE, BPA, and LAS were determined using the fully automated system with anti-APE, BPA or LAS Ab-BMPs (Fig. 4). The dose–response curves were obtained for the values of B/B_0. The detection ranges for APE, BPA, or LAS was in the range of 6.6 ppb–66 ppm, 2.3 ppt–2.3 ppm and 35 ppt–35 ppm, respectively. The sensitivity of the assay was determined as the limit of detection, defined as the lowest measurable concentration of antigens (APE, BPA, and LAS) that could be distinguished from zero analytical concentration. The detection limits were compared with these of ELISA for various EDCs [9,10,24,25], GC–MS [26,27] and LC–MS [24] for APE, BPA and LAS (Table 1). The presented system gave wider range and lower detection limit than ELISA in which the same antibodies were used for detection. Furthermore, the similar or lower detection limits were obtained as compared with recent reports in competitive immunoassay for EDCs, such as BPA (2 ppb) [24] and polychlorinated biphenyls (5 ppb) [25]. Detection limits of LAS and BPA were at similar levels compared with these of GC–MS or LC–MS.

It is well acknowledged that there are several advantages in immunoassay using magnetic particles as

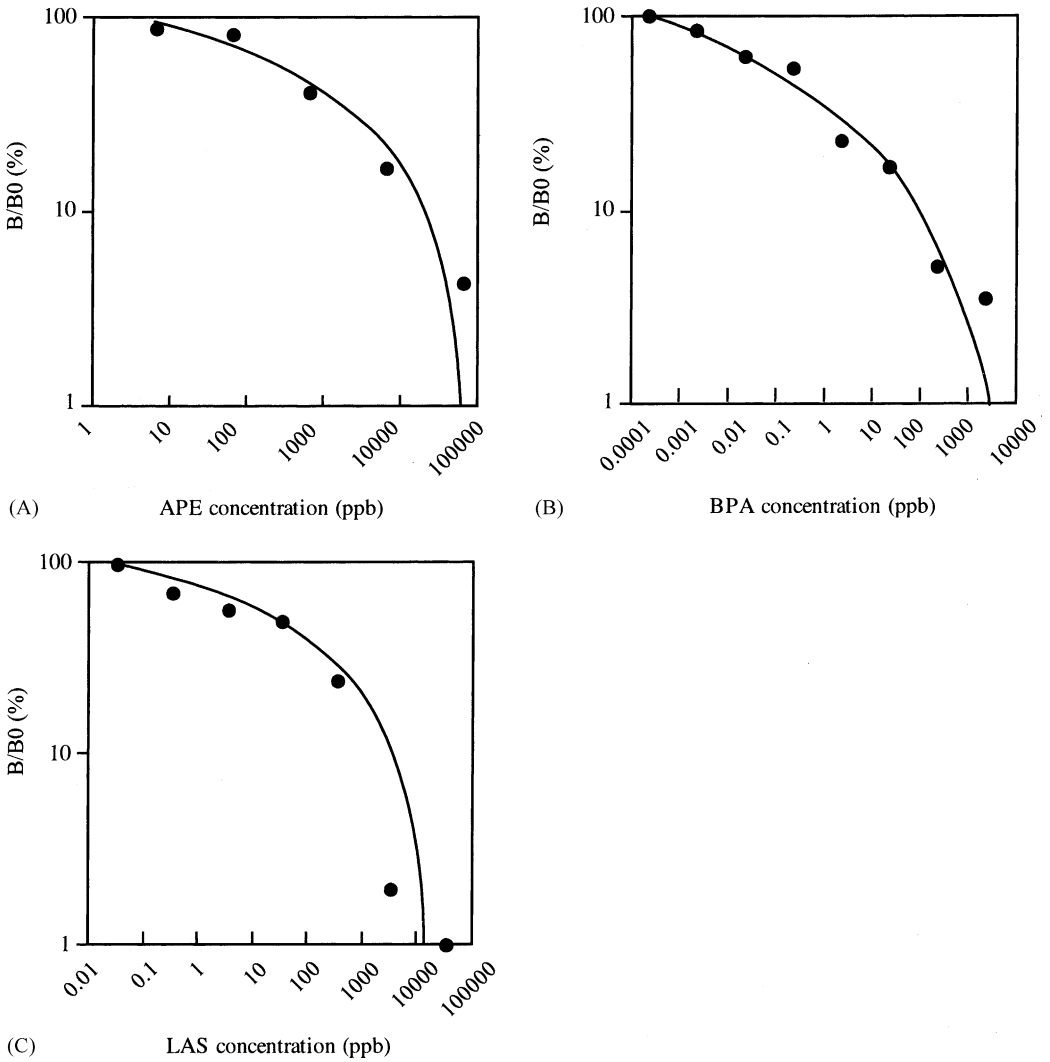

Fig. 4. Dose–response curves for APE (A), BPA (B) and LAS (C). ALP-EDCs concentration, Ab-BMPs concentration and luminescence substrate concentration were 5, 500 μg/ml and 330 μM, respectively. Antigen–antibody reaction time was 10 min. The total reaction volume was 100 μl.

Table 1
Detection limits of fully automated system, ELISA and GC–MS or LC–MS

Antigen	Fully automated system	ELISA (microtiter plate) (ppb)	GC–MS (or LC–MS) (ppt)
APE	66 ppb	50	25
BPA	2.3 ppt	2.0	100 (LC–MS)
LAS	35 ppt	20	20

compared with ELISA in which either antibodies or antigens directly adhere to polystyrene or polyvinyl microplates [28]. Potentially larger surface areas on the particles may provide higher density of antibody on their surface. When antibody was conjugated to AMPs, monodispersed AMPs (40–480 nm) were only 25%, and the average diameter was 835 nm. In contrast, the percentage of monodispersed BMPs (40–200 nm) was 44%, and the average diameter was 384 nm. The detection limit of BMP-based immunoassay was 10^3 times higher than that with AMP-based immunoassay. Detection limits were dependent on particle dispersion, resulting in enhanced reaction surface.

3.4. Specificity of monoclonal antibodies

Table 2 shows the cross-reactivity of anti-APE, BPA and LAS antibodies. Anti-APE antibody cross-reacted with not only nonylphenol with various ethylene oxide regions (*n*: 2–20), but also with nonylphenol itself. The antibody did not cross-react with BPA. The cross-reactivity of anti-LAS antibody with LAS analogues such as 4-ethylbenzene sulfonate and 4-*n*-octylbenzene sulfonate was low, and sodium dodecyl sulfate (SDS), which has the same chemical property as LAS was slightly recognized at 4% of cross-reactivity. However, it reacted with nonylphenol at 34% of cross-reactivity. Anti-BPA cross-reacted with a structural related compound of BPA, 4,4′-dihydroxy diphenyl, by 25%. Anti-APE antibody specifically reacted with the ethoxy regions, anti-BPA antibody with the phenol region, and anti-LAS antibody with the alkyl regions and phenol region.

Table 2
Cross-reactivity of antibodies with various chemicals

Antibody	Chemicals	Cross-reactivity (%)
Anti-APE	Alkylphenol ethoxylates[a] (ethoxy region: *n*)	
	$n = 0$ (alkyllphenol)	31
	$n = 2$	49
	$n = 10$	100
	$n = 20$	101
	Linear alkylbenzene sulfonates	
	$n = 12$	–
	BPA	–
Anti-LAS	Linear alkylbenzene sulfonates (C in alkyl region: *n*)	
	$n = 2$	7
	$n = 10$	10
	$n = 20$	100
	Nonylphenol	34
	Sodium dodecyl sulfate	4
Anti-BPA	Bisphenol A	100
	4,4′-Dihydroxy diphenyl	25
	17β-Estradiol	–

Standard: APE, alkylphenol ethoxylate ($n = 10$); LAS, alkylbenzene sulfonates ($n = 12$); BPA, bisphenol A.

[a] Nonylphenol ethoxylates were used as alkylphenol ethoxylates.

4. Conclusions

EDCs, such as APE, BPA and LAS at ppb or ppt levels have estrogenic effects in vivo and in vitro [3,4,7,8]. However, there are no government approved and standardized protocols for measuring EDCs. Therefore, a sensitive and rapid analysis system for measuring EDCs is needed. Lower detection limits were successfully achieved by using a fully automated immunoassay system with Ab-BMPs. Our results suggest that APE, BPA and LAS in natural environments can be measured directly without concentrating samples.

Future studies which entail the determination of emission standards from numerous environmental samples and the observation of how wildlife and ecosystems are affected can be easily accomplished through this fully automated immunoassay system. Advantages therefore of this system compared with GC–MS or LC–MS method include the ability to examine multiple samples in a single assay. Furthermore, the detection methods are very simple requiring no complicated instrumentation. It would be possible to extend this approach to other pollutants present in environmental samples.

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