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Flatfish Vitellogenin Detection Using Optical Waveguide Lightmode Spectroscopy-based Immunosensor

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Abstract A sensitive optical waveguide lightmode spectroscopy-based immunosensor was developed to detect vitellogenin in seawater flatfish (*Paralichthys olivaceus*). For this purpose, anion-exchange column chromatography with DE-52 resin was used to purify flatfish vitellogenin from flatfish serum containing vitellogenin that had been induced using an intraperitoneal 17β-estradiol injection. The anti-flatfish vitellogenin antibody used as the biological component of the above immunosensor was prepared using the purified flatfish vitellogenin. The change in the incoupling angle according to the complexation between the flatfish vitellogenin and its antibody, immobilized over an optical grating coupler sensor chip, was measured to calculate the sensor response. The immunosensor was quite specific to flatfish vitellogenin binding, based on no sensor response in the case of bovine serum albumin immobilization. When plotted using double-logarithmic scales, the sensor responses increased linearly in flatfish vitellogenin concentrations of 0.00675–67.5 nM, with a detection limit of 0.0675 nM. The reusability during seven repetitive measurements was reasonably fair for the preliminary screening of flatfish vitellogenin.

Keywords: Optical waveguide lightmode spectroscopy-based immunosensor, sensitive detection, flatfish vitellogenin

Endocrine disruptors are exogenous substances that cause adverse biological effects by interfering with the endocrine system and disrupting the physiological function of hormones [25]. Some well-known examples include 17β-ethinylestradiol (the contraceptive pill), dioxins, PCBs, PAHs, furans, phenols, and several pesticides (most prominent DDT and its derivatives) [25]. Because of recent environmental contamination, the discharge of these chemicals into rivers and oceans is now resulting in possible accumulation in the human body through the food chain, thereby potentially leading to harmful effects on the human body by triggering the above-mentioned changes. As fish are positioned in the upper part of the food chain, they represent an important route of human contamination with endocrine disruptors, thus stringent surveillance of edible freshwater and seawater fish is required against possible contamination with endocrine-disrupting chemicals [6].

It has been reported that vitellogenin, a potent fish biomarker that is an estrogen-dependent egg yolk protein precursor and phospholipoglycoprotein in nature, is known to be secreted in enormous amounts (one-million-fold increase, maximum) into the blood of most fish, including carp, flatfish, and sea bream, exposed to endocrine disruptors, owing to an abnormal induction of the vitellogenin gene [1, 27]. As a result, the contamination of fish with these chemicals can be determined by measuring the level of vitellogenin in the blood.

Thus, a rapid and sensitive screening tool that can efficiently measure the level of vitellogenin in fish serum is urgently needed for the effective assessment of endocrine-disruptor contamination in fish, thereby protecting the quality of fish consumed by the domestic market. A label-free immunosensor that does not require a probe molecule for signal transduction and can measure changes in physical parameters, such as frequency or a refractive index, caused by an immune response [14, 16, 19], may be a strong candidate for on-site vitellogenin detection, owing to its simple measurement procedures, inertness to interferences normally found in the case of a labeled immunosensor and ELISA, and real-time properties [3, 4, 7].

An optical waveguide lightmode spectroscopy (OWLS)-based biosensor was recently developed in the field of integrated optics, and exploits the science of light guided in structures smaller than the wavelength of the light [29]. This also has enormous potential as a label-free device for measuring changes in the refractive index in affinity-based processes in bio- and nanotechnologies [22–24]. The present authors previously developed an OWLS-based immunosensor that can detect *Salmonella* spp. with a limit

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of detection (LOD) of 1.3×10^3 CFU/ml [3]. Therefore, from this result, it can be inferred that the above immunosensor can also be applied to preliminary vitellogenin screening in fish.

Accordingly, this is the first report on a sensitive measurement system for vitellogenin in flatfish (*Paralichthys olivaceus*), a seawater species widely consumed in Korea, using an OWLS-based immunosensor with an optical grating coupler (OGC) and anti-flatfish vitellogenin antibody made from specially prepared flatfish vitellogenin as the transducer and biological component, respectively.

MATERIALS AND METHODS

Reagents

3-Aminopropyltriethoxysilane (APTS) and glutaraldehyde were used for the silanization of the OGC sensor chip and activation of the silanized sensor chip, respectively, during the antibody binding and were purchased from (Sigma-Aldrich Co., St. Louis, MO, U.S.A.). The 17β-estradiol, peanut oil, and protease-inhibitor cocktail, required for the induction of vitellogenin in the flatfish, were also obtained from Sigma Chemical Co., along with the reagents needed for the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified flatfish vitellogenin, such as acrylamide, Coomassie Brilliant Blue R-250, and a standard mixture for molecular masses of 30–200 kDa, including carbonic anhydrase (29 kDa, from bovine erythrocytes), ovalbumin (45 kDa, from chicken egg white), phosphorylase b (97 kDa, from rabbit muscle), β-galactosidase (116 kDa, from *Escherichia coli*), and porcine myosin (200 kDa). A preswollen and microgranular cellulose anion-exchange resin with the diethylaminoethyl tertiary amine functional group DE 52 was the product of Whatman, Inc. (Florham Park, NJ, U.S.A.). A Horseradish Peroxidase-Conjugated Goat Anti-Mouse IgG Solution (Fc Fragment Specific) and TMB Conductivity One-Component HRP Microwell Substrate, containing 3,3',5,5'-tetramethylbenzidine (TMB) in a mildly acidic buffer were used for the ELISA to determine the reactivity of the prepared antibody, and were the products of Pierce Biotechnology, Inc. (Rockford, IL, U.S.A.) and BioFX Laboratories (Owings Mills, MD, U.S.A.), respectively. All the other chemicals were the products of Sigma Chemical Co. or guaranteed reagents from various suppliers, plus double-distilled water was used throughout the study.

Transducer

The OGC sensor chip (OW 2400) was obtained from MicroVacuum Ltd. (Budapest, Hungary), and its diffraction grating had a surface relief depth of ~20 nm, grating periodicity of 2,400 lines/mm, and grating area dimensions of ~2 mm (length) and 16 mm (width). The refractive index (n_F) and thickness (d_F) of the waveguide layer under the diffraction grating were 1.77 and 170–220 nm, respectively. The dimensions of the substrate glass slide under the waveguide layer were 48 mm (length), 16 mm (width), and 0.55 mm (thickness), plus a refractive index (n_S) of 1.53.

Preparation of Flatfish Vitellogenin and Its Antibody

Vitellogenin was induced in the flatfish as follows, according to the method of Moon *et al.* [12] with a slight modification. A few adult flatfish in good physical condition were adapted to a breeding basin, containing seawater adjusted to 16°C, for one week. During this period and the following breeding period, the initial dissolved oxygen level was maintained with an air pump installed inside the breeding basin. After one week, half of the flatfish was transferred to a small basin containing a 0.01% ethyl 3-aminobenzoate anesthetic for 5 min. A hormone solution that had been prepared by dissolving 80 mg of 17β-estradiol in 5 ml of peanut oil was then intraperitoneally injected into the anesthetized flatfish at a dosage of 1 mg per 100 g fish weight. Thereafter, the treated flatfish were transferred back to the breeding basin, where they remained for another week. A boosting injection was performed in the same way as described above. One week after the booster injection, blood was collected from the treated flatfish using a syringe into a test tube, followed by the addition of a protease-inhibitor cocktail (Sigma Chemical Co.), containing sodium EDTA, 4-(2-aminoethyl)benzenesulfonyl fluoride, E-64, leupeptin, aprotinin, and bestatin, to inhibit the proteolysis of the vitellogenin. After 30 min at room temperature, the flatfish serum was prepared by centrifugation at 4,000 rpm for 30 min. The induction of vitellogenin in the retrieved flatfish serum was confirmed by 7% SDS-PAGE. The remaining untreated flatfish without the 17β-estradiol injection were used as the control and maintained in the breeding basin, and then bled and analyzed for their vitellogenin level using 7% SDS-PAGE, like the treated flatfish.

The flatfish vitellogenin was purified by anion-exchange column chromatography, utilizing the DE-52 resin, as reported previously [12]. However, a linear NaCl gradient up to 0.25 M in an eluting buffer solution of 50 mM Tris-HCl (pH 8.0) was also employed in this study.

To prepare the anti-flatfish vitellogenin antibody, the purified flatfish vitellogenin from the above column chromatography was separated again using 7% SDS-PAGE. The developed polyacrylamide gel was then stained with 0.1% Coomassie Brilliant Blue R-250 for 30 min, followed by destaining with 10% methanol in 10% glacial acetic acid overnight. Thereafter, the destained gel with the flatfish vitellogenin band was cut off, frozen in liquid nitrogen, lyophilized, and finally crushed. The crushed gel was then suspended in 600 μl of PBS (100 mM NaCl,

3 mM KCl, 2 mM KH_2PO_4, and 100 mM Na_2HPO_4) to recover the antigen. The preparation of the anti-flatfish vitellogenin antibody was undertaken according to a previous report and the reactivity of the prepared antibody confirmed by ELISA, as described below, and Western blotting [12].

The ELISA was undertaken for the prepared antibody and control serum as follows. The purified flatfish vitellogenin solution, diluted with the ELISA coating buffer (0.032 M Na_2CO_3 and 0.068 M $NaHCO_3$) to 250 ng/well, was added to a 96-well plate in 50 µl, followed by incubation of the plate for 2 h at 37°C. After discarding the coating solution, 200 µl of 2% skim milk was dispensed into each well of the plate to block the unoccupied surface for 30 min at 37°C. After washing the plate once with TBS-T (10 mM Tris, 150 mM NaCl, and 1% Tween 20), 50 µl of the antibody solution or control serum at each dilution was added into each well of the plate, and allowed to react for 2 h at 37°C. After washing the plate three times with TBS-T, 50 µl of a Horseradish Peroxidase-Conjugated Goat Anti-Mouse IgG Solution (Fc Fragment Specific) diluted 5,000-fold was dispensed into each well of the plate, and the plate left to react for 1 h at 37°C. Finally, the plate was washed five times with TBS-T, followed by the addition of 50 µl of the TMB Conductivity-One Component HRP Microwell Substrate to each well. Ninety seconds after the color change, 100 µl of 0.05 M H_2SO_4 was added to each well in the plate to stop the reaction. The absorbance at 450 nm was then measured using a microplate reader.

Arrangement of Immunosensor System

The OWLS-based immunosensor system used in this study was operated in a direct-binding mode, as reported previously [3]. The system setup included a Reglo digital pump (ISM 832A, Ismatec Co., Switzerland), injector (Rheodyne 7225, Supelco Co., St. Louis, U.S.A.), the main unit (OWLS 110, MicroVacuum Ltd.) composed of a sensor holder attached to a flow-through cuvette over the OGC sensor chip, two photodiodes, a beam mirror, shutter, and He-Ne laser source emitting a monochrome light of 632.8 nm, plus a PC. The individual parts of the system were connected with capillary tubing from Ismatec Co. During the operation, the monochrome light was diffracted by the optical grating of the sensor chip and started to propagate *via* the total internal reflection inside the waveguide layer. At a well-defined incident angle, the phase shift during one internal reflection was equal to zero and a guided mode excited, which then generated an evanescent field penetrating into the covering medium [29]. The change in the refractive index at the surface, caused by the added layer resulting from the antibody-antigen complexation, was then monitored on-line by precise measurement of the incoupling angle (α) as a function of time using the operating software of the system.

Immobilization of Antibody

The anti-flatfish vitellogenin antibody was immobilized *in situ* over the surface of the OGC sensor chip according to the APTS protocol of Kim *et al.* [3], owing to its higher concentration dependency, resulting in a good LOD, baseline stability, and simple procedure of antibody coating. However, in this study, the antibody solution was diluted with the reaction buffer of the system, 4 mM Tris-HCl (pH 7.2), within a range of 1:1,000–1:10 before the immobilization.

Analytical Procedure

Five-hundred µl of different concentrations of the flatfish vitellogenin solution was separately added to the immunosensor system in a flow mode at a flow rate of 200 µl/min, followed by elution of the reaction buffer. A sensor response was regarded as a surface mass change (ng/cm^2), caused by the flatfish vitellogenin, in the reaction buffer before and after a sample injection. A change in the surface mass during a measurement was automatically displayed on the computer screen using the operating software, based on the measured incoupling angles comprising the transverse electric mode (α_{TE}) and transverse magnetic mode (α_{TM}). The regeneration of the OGC sensor chip was performed in the flow mode by the addition of 500 µl of 10 mM HCl after each measurement.

RESULTS AND DISCUSSION

Flatfish are one of the major breeding species for shallow-sea farming in Korea, yet the possibility of contamination with endocrine disruptors from the elevated burden of organic pollutants often discharged in coastal areas is a serious concern. In turn, this also implies that excessive levels of vitellogenin, an important biomarker protein for estrogenic compounds [1, 27], may be present in the blood of flatfish reared in coastal areas. Therefore, this study attempted to develop a rapid and sensitive OWLS-based immunosensor for screening flatfish vitellogenin in real-time.

Preparation of Flatfish Vitellogenin and Its Antibody

As the first step to make the immunosensor, flatfish vitellogenin and the corresponding antibody were prepared as described in the Materials and Methods section, as neither was obtainable from a commercial source.

When analyzed using 7% SDS-PAGE, dense vitellogenin bands were found in the flatfish sera treated with 17β-estradiol, compared with faint bands in the case of the control flatfish (data not shown). Next, flatfish serum induced with vitellogenin was purified by anion-exchange column chromatography using DE-52 resin, and the elaborately prepared antigen solution used for the antibody production.

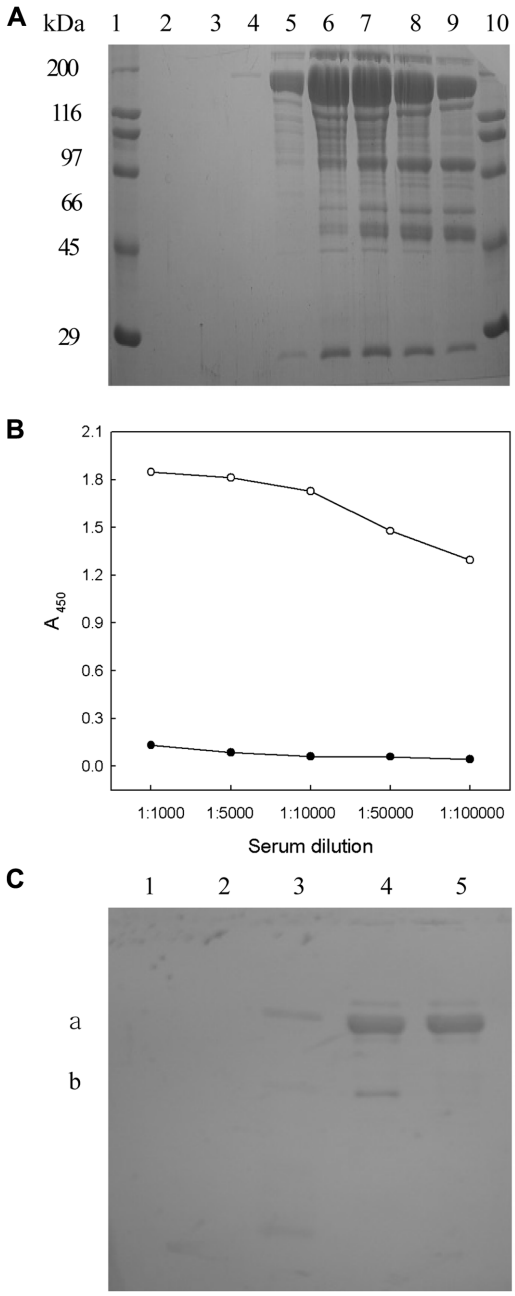

Fig. 1. SDS-PAGE electropherogram of purified flatfish vitellogenin using DE 52 column chromatography (**A**), ELISA binding graph of control serum and final anti-flatfish vitellogenin antibody from rabbits (**B**), and Western blotting for vitellogenin in flatfish sera (**C**).

A: lanes 1 and 10, standard mixture for molecular masses of 30–200 kDa; lane 2, elution E6; lane 3, elution E7; lane 4, elution E8; lane 5, elution E9; lane 6, elution E10; lane 7, elution E11; lane 8, elution E12; lane 9, elution E13. **B**: ○, final antibody; ●, pre-immune serum. **C**: lanes 1-5, Western blotting using antibody against purified flatfish vitellogenin; lane 1, standard mixture of molecular mass markers; lane 2, 250-fold diluted serum from male flatfish without induction; lane 3, 250-fold diluted serum from female flatfish without induction; lane 4, 700-fold diluted serum from female flatfish induced with 17β-estradiol dissolved in peanut oil; lane 5, 700-fold diluted serum from male flatfish induced with 17β-estradiol dissolved in peanut oil.

The individual column fractions of the 7% SDS-PAGE electropherogram in Fig. 1A show dense vitellogenin bands between the two molecular mass markers of 116 and 200 kDa, at an approximate position of 180 kDa [13]. Thus, since vitellogenin is reportedly known to be present in a dimeric form with a molecular mass around 370–450 kDa [10], the 180 kDa band found in this study may represent monomeric flatfish vitellogenin. Fig. 1B indicates the reactivities of the final anti-flatfish vitellogenin antibody to the antigen at various serum dilutions, which were measured by ELISA at 450 nm, together with those of the diluted control serum. Contrary to the first and second antibodies that showed conspicuously decreased absorbance values at 0.18 and 0.40 with a higher serum dilution (data not shown), the final antibody still exhibited a considerable reactivity of around 1.30 towards the antigen, even at a serum dilution of 1:100,000. When determined by Western blotting (Fig. 1C), two dense bands (band a), representing antibody-antigen complexation and with the same mobility, appeared in the sera of a female and male flatfish induced with 17β-estradiol. Band b found in the serum of the female flatfish induced with 17β-estradiol was presumed to be an immature vitellogenin isoform. Therefore, from these results, it was concluded that the purified flatfish vitellogenin and its antibody could be used as the analyte and biological component in the following immunosensor development [12].

Specificity of Immunosensor to Flatfish Vitellogenin Binding

To operate the OWLS-based immunosensor system, a Tris-HCl buffer solution with a molarity of 4 mM was selected, owing to its compatibility with the OGC sensor chip [11]. When time-dependent responses were measured at different pH values, the surface coverage increased abruptly during the initial phase of elution, and then converged to individual steady-state values (data not shown). Based on the resulting steady-state surface coverage values and baseline stability, 4 mM Tris-HCl (pH 7.2) was selected as the reaction buffer for the remainder of the study.

The specificity of the immunosensor in measuring flatfish vitellogenin was evaluated by injecting 6.75 nM of the purified flatfish vitellogenin into the system and comparing the changes in the surface coverage of the OGC sensor chips immobilized with the anti-flatfish vitellogenin antibody and an irrelevant protein with respect to the immune response of this study, bovine serum albumin (BSA) (Fig. 2). As depicted in Fig. 2, no sensor response was found in the case of BSA immobilization. However, the response of the antibody-coated sensor chip after injection with flatfish vitellogenin was evident, amounting to 35.0 ng/cm^2, thereby confirming the high specificity of the immunosensor to flatfish vitellogenin binding [3, 5].

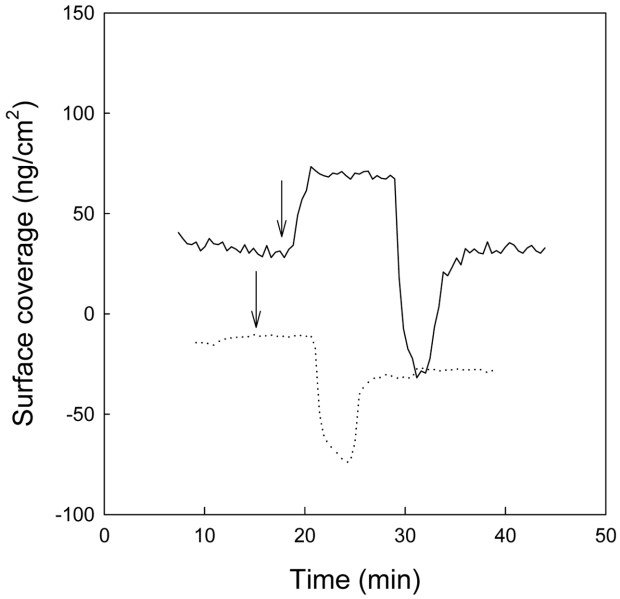

Fig. 2. Comparison of time-dependent surface coverage profiles of OWLS-based immunosensor detecting flatfish vitellogenin.
—, Sensor surface immobilized with anti-flatfish vitellogenin antibody; ·····, sensor surface immobilized with bovine serum albumin. Arrows indicate points of sample injection.

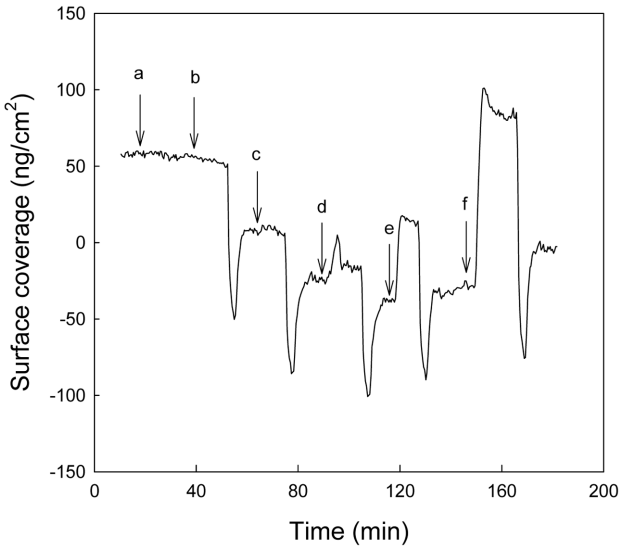

Fig. 3. Concentration-dependent responses of OWLS-based immunosensor detecting flatfish vitellogenin at anti-flatfish vitellogenin antibody dilution of 1:10 for immobilization.
Five-hundred μl of the reaction buffer (a) and flatfish vitellogenin solutions at concentrations of 0.00675 (b), 0.0675 (c), 0.675 (d), 6.75 (e), and 67.5 nM (f) was individually injected into the system after baseline stabilization, following 10 mM HCl treatment.

Concentration Dependency of Immunosensor According to Antibody Dilution

The responses of the immunosensor were measured at flatfish vitellogenin concentrations of 0.00675–67.5 nM and antibody dilutions of 1:1,000–1:10, after regenerating each step with 10 mM HCl. Fig. 3 shows the typical concentration-dependent response of the immunosensor prepared with an antibody dilution of 1:10. The sensorgrams for over 0.00675 nM flatfish vitellogenin were characterized by an initial steep increase, followed by a gradual decrease in the surface coverage to a steady-state value, which seemed to indicate a washing of the unbound flatfish vitellogenin molecules. This kind of behavior is typical for sensorgrams obtained using flow-type optoelectronic biosensors based on affinity binding [2, 3, 9]. In the present study, the time required to obtain a sensor response was around 10 min, irrespective of the analyte concentration. Like the injection of the reaction buffer itself (arrow a), no sensor response was found when the analyte concentration was 0.00675 nM (arrow b). The three-fold value against the standard deviation for baseline drift, which is normally accepted as the criterion for determining the LOD of an analytical method, was found in the range of 0.024–0.330 ng/cm^2. Consequently, the LOD of the immunosensor at an antibody dilution of 1:10 was safely presumed as 0.0675 nM flatfish vitellogenin. The sensor responses for each flatfish vitellogenin concentration over 0.0675 nM increased gradually according to the decrease in the antibody dilution. Thus, based on the above criterion, 0.675 nM flatfish vitellogenin was assumed as the LOD of the immunosensor at antibody dilutions of 1:1,000 and 1:100. When compared with a quartz crystal microbalance immunosensor, which has a LOD of 0.4864 nM for carp vitellogenin [6], the current immunosensor was found to have a similar or better sensitivity. Notwithstanding, although the sensitivity of the direct-binding OWLS-based immunosensor used in this study for flatfish vitellogenin detection was comparatively lower than that of a radioimmunoassay and ELISA [13, 15, 20], it can still be used as a potent screening tool for vitellogenin induced in the blood of fish contaminated with estrogenic compounds, such as 17β-estradiol and nonyl phenol, since vitellogenin induction is expected to occur at more than several thousand fold compared with that found in the control serum [26]. In fact, in a previous study by the present authors, the label-free immunosensor used in this study was able to measure the concentration range of vitellogenin in a control and 17β-estradiol-treated sera from adult male and female carp (data not shown).

When the relationship between the flatfish vitellogenin concentration and the surface coverage change at various antibody dilutions for immobilization was plotted in semi-logarithmic scales, typical first-order kinetic profiles were obtained (data not shown). Thereafter, linear relationships encompassing whole-flatfish vitellogenin concentrations were investigated and found using double-logarithmic scales, as reported previously [8, 18, 28]. For the linear equations in Fig. 4, the correlation coefficients (r) were in

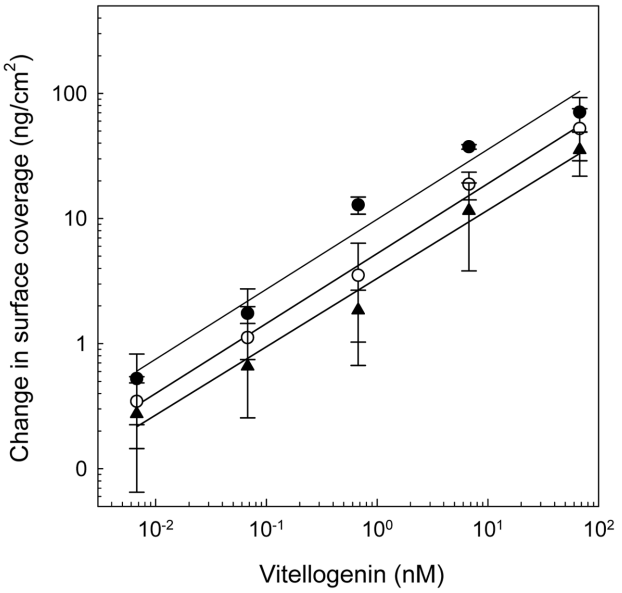

Fig. 4. Calibration curves for flatfish vitellogenin, plotted using double-logarithmic scales. Anti-flatfish vitellogenin antibody dilutions for immobilization: ▲, 1:1,000; ○, 1:100; ●, 1:10.
The correlation coefficients at the antibody dilutions of 1:1,000, 1:100, and 1:10 were 0.9838, 0.9848, and 0.9756, respectively. The measurements were taken in duplicate and error bars inserted.

the range of 0.9756–0.9848. Thus, based on the LODs and degree of the sensor responses, for the remainder of this study, the anti-flatfish vitellogenin antibody was diluted with the reaction buffer using a ratio of 1:10 before immobilization.

Reusability of Immunosensor

After repeated use, the immunosensor response gradually decreased, mainly due to partial loss of the bound antibody layer [17, 21]. In a previous report by the current authors, 10 mM HCl was quite effective as a regenerating solution for the repeated use of an OGC immunosensor detecting *S. typhimurium*, an important hygienic index in the food industry [3]. Therefore, 10 mM HCl was also used as the regenerating solution for the OWLS-based immunosensor used in this study.

To evaluate the reusability of the immunosensor, a flatfish vitellogenin solution with a concentration of 6.75 nM was injected into the immunosensor system seven times after regenerating the OGC sensor chip with 10 mM HCl. In this instance, the mean surface coverage change with the seven repetitive measurements was 27.51± 2.95 ng/cm^2, representing 85.25% of the first sensor response measured at 32.27±0.24 ng/cm^2. Nonetheless, even though the coefficient of variability of 10.72%, after repetitive use of the immunosensor, was not satisfactory for the quantification of flatfish vitellogenin, the immunosensor can still be reused for an initial screening of vitellogenin induced in the blood of flatfish contaminated with endocrine disruptors, since the stringent reproducibility needed for quantification is not normally required and the vitellogenin amplification is generally enormous [17, 26]. It is expected that the reproducibility for the detection of flatfish vitellogenin will be improved by ensuring uninjured antibody layers with the use of seven new OGC sensor chips immobilized with the anti-flatfish vitellogenin antibody.

Consequently, from the above results, the OWLS-based immunosensor in this study would appear to be a promising screening tool for the initial monitoring of possible flatfish contamination with endocrine disruptors in a flatfish farm. However, further research on vitellogenin monitoring using the developed immunosensor is required to evaluate the vitellogenin level in the blood of flatfish treated with endocrine disruptors, such as nonyl phenol.

Acknowledgment

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