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An optical immunosensor for rapid vitellogenin detection in plasma from carp (*Cyprinus carpio*)

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Abstract

Vitellogenin (vtg) has proven to be a sensitive and simple biomarker for assessing exposure of fish to environmental estrogens. The aim of this work was to develop a rapid, in the order of minutes, screening method for the detection of fish vtg. The surface plasmon resonance technique (Biacore X^{TM}) was coupled with immunodetection for the determination of fish vtg in plasma and mucus from carp (*Cyprinus carpio*). Monoclonal anti-vtg antibodies were linked on the sensor surface through chemical cross-linking via a capturing antibody. A simple regeneration process allowed the reuse of the sensor surface. Sensor optimisation was carried out using carp vtg. The developed immunosensor was tested with vtg spiked samples and with plasma and mucus from fish exposed to 17β-estradiol (E2). Vitellogenin could be detected in the ppm range in buffer as well as in plasma and mucus. Good discrimination between control and exposed samples was obtained. The results were compared with ELISA and a correlation coefficient of $R^2 = 0.85$ ($n = 9$) between the two methods indicated that the immunochemical biosensor could be used for the analysis of vtg in fish plasma samples. The assay time was 20 min hence allowing for rapid sample screening.
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Keywords: Optical immunosensor; Vitellogenin; Surface plasmon resonance; Carp

1. Introduction

There is growing evidence and concern of the impact of endocrine disrupting chemicals (EDCs) in the environment. Endocrine disruptors are exogenous agents that alters functions of the endocrine system consequently causing adverse effects of the reproductive health of organisms [1]. A large range of EDCs have been identified, making the detection of a single compound or group of compounds difficult. Consequently, the development of analytical techniques using specific biomarkers have shown to be a promising route for evaluating EDC contamination in the aquatic environment. Among these, the detection of the phospholipoglycoprotein vitellogenin (vtg) has been commonly reported.

vtg is produced as the yolk protein precursor in the liver of oviparous vertebrates such as fish. The transcription of the vtg gene takes place as a result of circulating 17β-estradiol (E2) produced in the gonads of female fish during sexual maturation. The E2 enters the liver cells by diffusion and is retained in the target cells through binding to a specific receptor protein (estrogen receptor) hence inducing the activation of the vtg loci [2]. Although naturally present only in female fish, the expression of the protein has been observed to be induced in male and immature fish through the exposure to xeno-estrogens [3]. Consequently, the presence of vtg in plasma and sera of male and sexually immature fish has proven to be a sensitive and simple biomarker for assessing exposure to environmental estrogens [4–6].

Several analytical approaches for vtg determination have been reported such as separation techniques and immunoassays. Allner et al. [7] used an electrophoretic method for the determination of estrogen-induced proteins in fish exposed to synthetic and naturally occurring chemicals. Similarly, Song et al. [8] reported about the separation and detection of vitellogenin in fish plasma by capillary zone electrophoresis. Chromatographic and mass spectrometry methods have also been described [9–11]. In addition a range of immunochemical methods such as enzyme linked immunosorbent assays (ELISAs), radioimmunoassay

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(RIA) and Western Blotting have been studied. For quantitative detection of vtg the most widely used techniques are based on heterogeneous competitive ELISAs or sandwich ELISAs. In the last decade a range of ELISAs for measuring vtg concentrations in various fish species have been developed, such as sole (*Solea vulgaris*) [12], striped bass (*Morone saxatilis*) [13], eelpout (*Zoarces viviparus*) [14], Artic charr (*Salvelinus alpinus*) [15], fathead minnow (*Pimephales promelas*) [16,17], rainbow trout (*Oncorhynchus mykiss*) [18], zebrafish (*Danio rerio*) [19], Japanese medaka (*Oryzias latipes*) [20] and carp (*Cyprinus carpio*) [21]. Despite showing high sensitivities, ELISAs used for vitellogenin detection are multistage processes with long overall analysis times varying from 1 to 4 days [22].

Biosensors have in recent years shown to represent interesting alternatives for developing rapid screening techniques. However, only very few studies report the development of biosensors for vtg detection. In Oshima et al. [23] the use of a quartz crystal microbalance for the determination of plasma vitelllogenin was described. Darain et al. [24] reported the detection of vitellogenin using impedance spectroscopy. The same author has also reported about a separation-free amperometric immunosensor for vitellogenin detection based on screen-printed carbon assays modified with a conductive polymer [25]. Despite demonstrating sensitivities in the ppm or sub-ppm vitellogenin range, these methods show long incubation times for the preparation of the sensor surfaces. Darain, et al. [25] reported a total time for the fabrication of the immunosensor array of 28 h. Similarly, long incubation times for the modification of the electrode surface used for impedance spectroscopy have been reported [24]. Likewise, the preparation of the quartz crystal microbalance involves long incubation times as well as large sample volumes [23].

The present study was aimed at developing an optical-label free sensor for rapid, in the order of minutes, vtg detection in fish. The surface plasmon resonance technique (Biacore X^{TM}) was coupled to immunodetection for the determination of fish vtg in plasma. The analytical parameters (specificity, sensitivity, reproducibility, analysis time, matrix effects) of the system were evaluated. Hence, the developed immunosensor was tested with vtg spiked samples and with plasma and mucus from fish exposed to E2. The obtained results were compared with the ELISA test used as a reference method.

2. Materials and methods

2.1. Reagents

Vitellogenin (vtg) from carp (*C. carpio*) was purchased from Biosense Laboratories A/S, (Bergen, Norway). The product had been obtained through the purification of plasma from 17β-estradiol-induced fish by selective precipitation with $MgCl_2$, in the presence of EDTA. Rabbit polyclonal antibodies (pab) (purified IgG fraction) against vitellogenin from carp and monoclonal mouse anti-carp vtg antibodies (mab) were also purchased from Biosense Laboratories (Bergen, Norway). *N*-Hydroxysuccinimide was from FLUKA (Milan, Italy), anti-mouse antibodies, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide, bovin serum albumin (BSA), sodium hydroxide (NaOH) and ethanolamine were obtained from Sigma (Milan, Italy). Saline phosphate buffer, PBS (0.1 M pH 7.0) was prepared from di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) and sodium chloride (NaCl) purchased from Merck (Milan, Italy). Acetate buffer (0.01 M, pH 5.0) was prepared from sodium acetate (CH_3COONa) and acetic acid (CH_3COOH) from Merck. The running buffer employed in all experiments was HBS-EP containing [4-(2-Hydroxyethyl)piperazine-1-ethanesulonic acid] (HEPES, 0.1 M), sodium chloride (0.15 M), polyoxyethylene-sorbital monolaurate (Tween 20, 0.005%) and ethylenediaminetetraacetic acid (EDTA, 3 mM) from Sigma. All aqueous solutions were prepared with Milli-Q water, obtained from a Milli-Q system (Millipore, Milford, USA). The running buffer was filtered (0.2 μm) and degassed before use.

2.2. Instrumentation

Surface plasmon resonance measurements were performed with the Biacore X^{TM} instrument (Biacore AB, Uppsala, Sweden). The measurements were carried out using CM5 chips (carboxymethylated dextran covalently attached to a gold surface) with HBS-EP as running buffer (5 μl/min). The SPR signal was expressed in resonance units (RU).

2.3. Immobilisation of antibodies

2.3.1. Capturing antibodies

Mouse monoclonal anti-carp vtg antibodies (mab) were immobilised with anti-mouse as capturing antibodies. The sensor chip was activated using a solution of *N*-hydroxysuccinimide (NHS) (50 mM) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDAC) (200 mM) in water [26]. The solution was passed over the sensor surface for 7 min at a flow rate of 5 μl/min. Thereafter the anti-mouse antibodies were allowed to flow over the chip surface (50 μg/ml in acetate buffer, 15 min interaction time). Remaining active sites were blocked using ethanolamine (1 M). Hence, a mab solution 18 μg/ml in phosphate buffer (PBS, 0.1 M, pH 7.0) was allowed to flow over the sensor surface (interaction time 15 min).

2.3.2. Capturing antibodies with cross-linking

The mouse monoclonal anti-carp vtg antibodies (mab) were immobilised using cross-linking with anti-mouse antibodies. Firstly, the activation of the sensor surface was carried out as described in Section 2.3.1. Hence, the mouse-monoclonal anti-carp vtg antibodies (mab) were immobilised with anti-mouse as capturing antibodies. In order to achieve cross-linking between the two types of antibodies a solution of NHS/EDAC was used. In this case aminocoupling between the carboxylic and aminogroups of the two types of antibodies was achieved. Subsequently remaining activated sites were blocked with ethanolamine (1 M).

2.4. Measuring assay

The measuring cycle consisted of the following steps; (i) surface with immobilised capturing antibodies (anti-mouse) binding the anti-vtg mab with running buffer 5 μl/min (baseline), then (ii) addition of the analyte vtg; (iii) washing with buffer to remove excess analyte; primary response recorded; hence (iv) surface regeneration by dissociation of the immunochemical reaction and return to the baseline.

2.5. Surface regeneration

For surface reuse it was necessary to return to the baseline. For this purpose the dissociation of the formed immunocomplex was achieved using sodium hydroxide (NaOH) at different concentrations (0.5–10 mM, 2 μl).

2.6. Quantitative ELISA analysis of vtg

Using a procedure modified from Specker and Anderson [22], a quantitative vtg ELISA was performed using the monoclonal carp vtg antibody (Biosense Laboratories). Purified carp vtg protein (Biosense Laboratories) was used to coat the plates and for preparation of the standard curve. Briefly, purified carp vtg was serially diluted to obtain standard concentrations between 8 and 1000 ng/ml. Standards and diluted samples were incubated for 1 h at 37 °C with an equal volume of the primary antibody (diluted 1:1000). Triplicate aliquots of standards and samples (200 μl) were added to 96-well microtiter plates previously coated with vtg (100 ng/ml overnight at 4 °C) and incubated for 1 h at 37 °C. The plates were washed with tween-phosphate buffered saline (TPBS) and a 1:1000 dilution of sheep anti-mouse peroxidaseconjugated secondary antibody (Gibco-Invitrogen Life Technologies, Carlbad, CA, USA) was added and incubated for 1 h at 37 °C. Levels of vtg in samples were measured colorimetrically at 492 nm using *o*-phenylenediamine dihydrochloride as substrate with a Microplate Reader Model 550" from Bio-Rad Laboratories (Richmond, CA, USA). vtg ELISA Absorbance values (expressed as optical density, OD) were converted to the proportion of antibody bound (B) expressed as a percentage in the zero standard by the following equation: $B(\%) = (\mathrm{OD} - \mathrm{NSB}/\mathrm{OD}_0 - \mathrm{NSB}) \times 100$ (where OD is the absorbance of a given sample or standard, OD_0 is the absorbance of the zero standard and NSB is the non-specific binding absorbance value). In evaluating the detection limit of the ELISA assay, the minimum amount of vtg that produced a response significantly different from OD_0 was estimated around 15 ng/ml. The linear range of the standards obtained from the sigmoidal curve was between 125 and 1000 ng/ml. The sample concentration was obtained from the sigmoidal curve at a sample dilution of 1:50,000 and 1:5000 for the mucus.

2.7. Fish samples

In this study carp (*C. carpio*) was selected as test species. The carp is a freshwater cyprinid present both in the wild and as farmed species. Adult carps were purchased from ErreCi (Pisa, Italy). The fish was stored in 500 l oxygenated fish tanks, supplied with fresh water in a closed recirculating system with controlled temperature (20 ± 1 °C). Fish were kept under normal laboratory illumination with a daily photoperiod of 12 h, and were acclimatised to laboratory conditions for 1 week. Procedures for the care and management of animals were performed in accordance with the provisions of the EC Council Directive 86/609 EEC, recognised and adopted by the Italian Government (DL 27.01.1992, no. 116). Blood was collected from the caudal vein of adult male carp ($t = 0$) and this represented the negative control samples. Mucus was collected by scraping the tail surface with a plastic spatula into microcentrifuge tubes. Hence, the fish were injected i.p. with 2.5 mg/kg body weight of 17β-estradiol (E2) dissolved in corn oil. On days 5, 10 and 14 ($t = 5$, 10 and 14 days, respectively) after the E2 injection, blood was collected from the caudal vein from three fish species ($n = 3$). Plasma was separated by centrifugation at $1500 \times g$ for 20 min (4 °C) and frozen (−80 °C) until further analysis was undertaken. The mucus was weighed and homogenized in one volume of PBS (pH 7.4). The homogenates were centrifuged at $10{,}000 \times g$ for 15 min at (4 °C), supernatants were collected and used for vtg analysis.

3. Results and discussion

3.1. Immobilisation using capturing antibodies

Direct immobilisation of the analyte specific antibodies showed an analytical detection range in the order of 10–100 ppm. For increased sensitivity of the sensors (0.1–10 ppm) the use of capturing antibodies was attempted since this strategy allowed to control the orientation of the specific anti-vtg antibodies (mab). The sensor was thereafter tested for its ability to recognise the relative antigen. The vtg standard was diluted in PBS. A calibration curve was obtained by subsequent injections of vtg without regenerating the surface by dissociating the immunocomplex. A linear range was observed between 1 and 10 ppm vtg ($R^2 = 0.98$, $y = 3.9x - 2.2$) demonstrating that the sensor was able to recognise the antigen. Specificity was tested by injecting BSA (100 ppm). No response was observed. The interaction time was 15 min. However, the process was tedious and expensive as it was not possible to regenerate the sensor surface after the calibration. The absence of the dissociation step was due to the fact that the linking between the capturing and mab antibodies is a reversible, non-covalent affinity binding. Hence, treatment of the mab/anti-vtg/vtg complex with a dissociating agent also caused the dissociation of the mab from the capturing antibodies. Consequently, requiring reimmobilisation of mab prior to further measurements. Subsequently, cross-linking between the capturing and analyte specific antibodies was attempted.

3.2. Immobilisation using cross-linking

By using NHS/EDAC cross-linking between the aminogroups and carboxylic groups of the capturing and anti-vtg antibodies was obtained. The cross-linking of the two types of

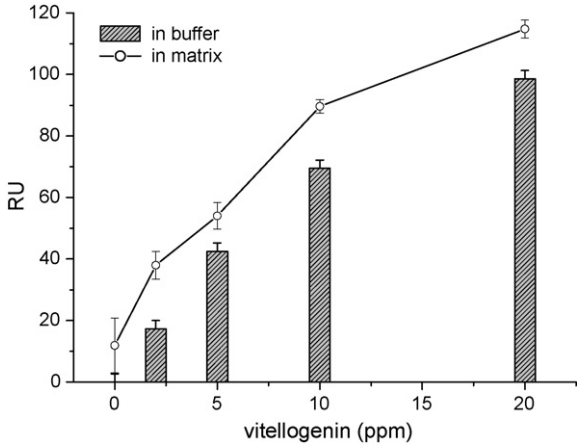

Fig. 1. Calibration curve of vitellogenin in buffer and in matrix (plasma, diluted 1:500).

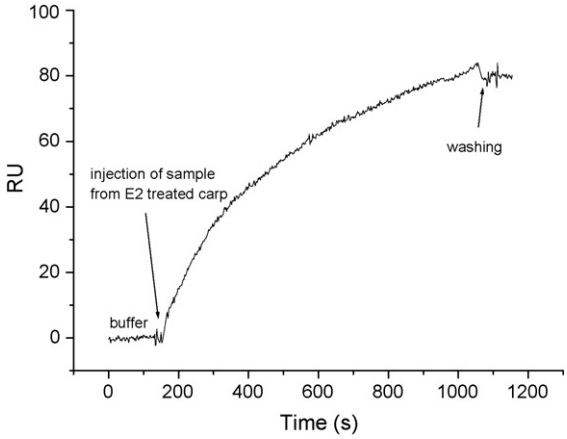

Fig. 2. Typical sensorgram recorded during the injection of a sample from an E2 treated carp.

antibodies significantly improved the method in terms of costs and analysis time since it was not necessary to reinject the mab anti-vtg (15 min). Moreover, it was possible to dissociate the mab/vtg complex leaving on the surface both the capturing and the mab antibodies. The amount of immobilised mab on the sensor chip surface was estimated from the RU signal obtained where 1000 RU represents a change in surface protein concentration of 1 ng/mm^2 (instrument handbook). In the present study the immobilisation of the mab anti-vtg antibodies resulted in a RU shift of 2220 ± 70 RU ($n = 3$), hence corresponding to a surface coverage of 2.2 ng/mm^2. Furthermore, the affinity constant between the vtg and the mab was evaluated using the Biaevaluation software following a 1:1 Langmuir association model. Three standard curves were used for the evaluation. The affinity constant K_D was found to be $(4.5 \pm 1.2)\ 10^{-10}$ M. Selectivity of the sensor was tested using BSA (100 ppm). No binding was observed demonstrating that the sensor was specific for the antigen. With this assay scheme a calibration curve was carried out in the ppm range. The binding between the immobilised mab and the vtg obey a Langmuir type adsorption isotherm. However, for quantification purposes, only the linear part of the sigmodial curve was considered. A linear range from 1 to 10 ppm was obtained ($R^2 = 0.98$, $y = 6.9x + 2.9$) with an average CV of 7% ($n = 3$ for each concentration). The limit of detection (L.O.D.) was estimated to be 1 ppm (three times the standard deviation of the blank).

3.3. Matrix effects

Since the applicability would be the vtg detection in fish plasma, the influence of this matrix on the signal was tested using plasma samples from fish not exposed to any estrogenic compounds (negative controls). Different dilutions as well as the effect of filtration were studied. The samples were diluted in PBS. Filtration using a 0.22 μm filter did not appear to have an effect on reducing aspecific adsorption. Despite the fact that the non-exposed fish (reference) resulted in an aspecific response it was possible to detect the presence of vtg (standard additions) to the matrix (Table 1). A calibration curve of the vtg in the matrix was thereafter carried out at a matrix dilution of 1:500. The RU signal obtained for the vtg at different concentrations in the matrix was comparable with that obtained in buffer (Fig. 1).

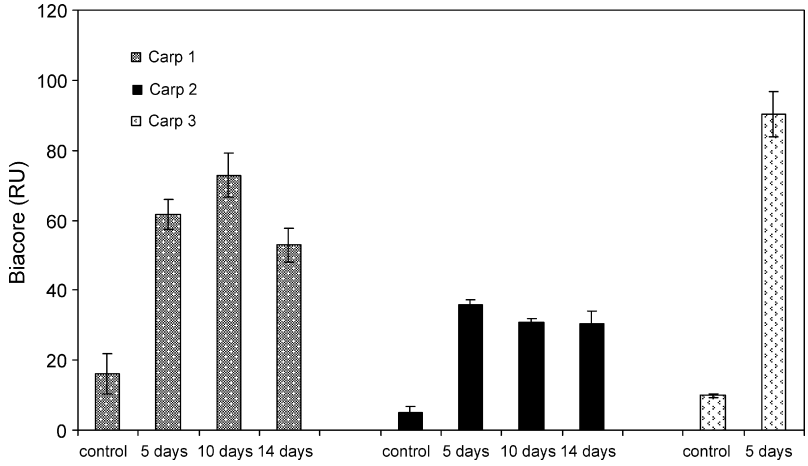

Fig. 3. Results of the analysis of control and E2 treated carp plasma samples.

Table 1
Plasma matrix effects

Dilution	Matrix (RU)	matrix + vtg 10 ppm (RU)	vtg signal/matrix
1:10	93 ± 9 (n = 3)	102	1.1
1:25	61 ± 10 (n = 3)	92	1.5
1:50	54 ± 10 (n = 5)	76	1.4
1:500	12 ± 9 (n = 3)	90 ± 2 (n = 3)	7.5

3.4. Analysis of plasma samples

The analysis of E2 exposed fish using the optical immunosensor was carried out using the previously described protocol. The samples were diluted 1:1000 in PBS. A typical sensorgram recorded during the injection of a sample from an E2 treated carp is shown in Fig. 2. Despite obtaining some aspecific absorption from the negative control samples, a good discrimination between the control and E2 treated fish could be obtained (Fig. 3). Furthermore, a good reproducibility was obtained with an average CV of 12.9% ($n = 10$). High RU values were observed for the sample obtained after 5 days of E2 treatment indicating a fast vtg response in the fish. The results were compared with ELISA. The sample concentrations were estimated from the ELISA sigmoidal curve (Fig. 4). For the immunochemical biosensor the sample concentration was estimated by subtracting the RU value of the control sample from the exposed sample and hence estimating the concentration from the vtg calibration curve. A relative good correlation between the two methods was obtained ($R^2 = 0.85$, $n = 9$) indicating that the immunochemical biosensor could be used for the rapid analysis of vtg in fish plasma samples.

3.5. Analysis of mucus samples

An interesting matrix for the non-invasive vtg detection is represented by mucus. The mucus can be obtained from the fish by simple scraping on the fish surface (see Section 2.7). Mucus was obtained from one carp not treated with any estradiol (negative control). Hence, after the fish had been treated with E2, mucus was sampled on 3 different days ($t = 5$, 10 and 14). The biosensor analysis was carried out as previously described. The mucus showed some matrix effects that improved using filtration. A good compromise between the lower expected vtg concentrations in this matrix [27] and the dilution factor was found to be 1:100. At this dilution the matrix was spiked with 10 ppm. A signal comparable to that in buffer could be obtained (Table 2). vtg could be detected in the mucus after 14 days (Fig. 5). Compared to the plasma it appeared that a longer time period is required for the vtg to be detectable in the mucus [27]. Despite being close to the detection limit of the sensor the results show that this matrix might offer interesting future possibilities for non-invasive vtg detection.

Table 2
Mucus matrix effects

Dilution	filtration	vtg (ppm)	RU
1:20	-	-	75
1:20	Yes	-	41
1:50	Yes	-	55
1:100	Yes	-	45
1:100	Yes	10	93
Buffer	Yes	10	91

3.6. Enhancing the primary response

To increase the sensitivity of the sensor, the use of polyclonal antibodies was employed [28]. The vtg is likely to have several epitopes and it is in this case possible to enhance the primary response using anti-vtg polyclonal anti-rabbit antibodies (pab). After measuring the vtg standard the secondary response is obtained (without regeneration of the surface) by adding pab directly to the bound vtg with subsequent washing of the surface to remove excess pab (Fig. 6). The amplification was effective as the primary response was increased by about 30%. Furthermore, the regeneration process remained a simple and fast step as the formed immunocomplex could be easily dissociated using sodium hydroxide at different concentrations for 30 s leaving on the sensor surface the capturing and anti-vtg antibodies. This type of enhancement could prove to be important for the analysis of matrices such as mucus where the expected vtg concentration is lower compared to that found in plasma.

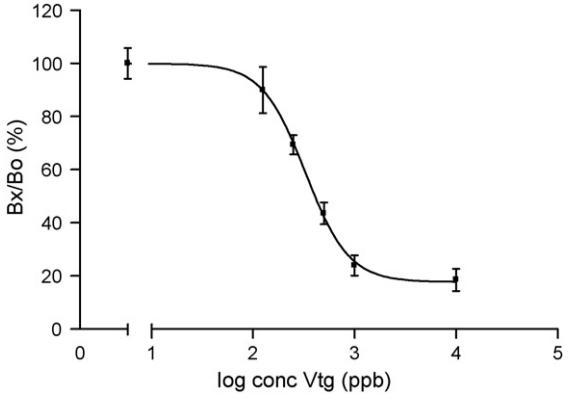

Fig. 4. Dose-response curve obtained by ELISA.

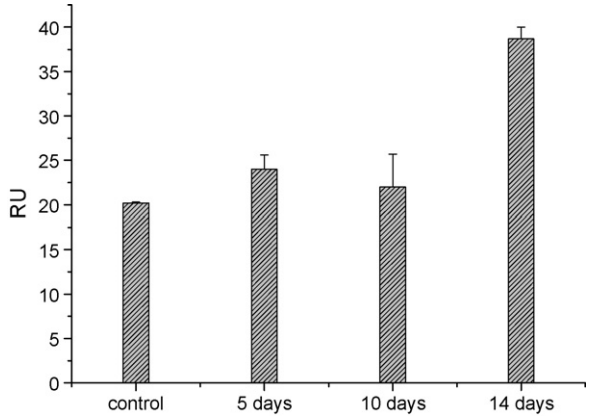

Fig. 5. Results of the analysis of control and E2 treated carp mucus samples.

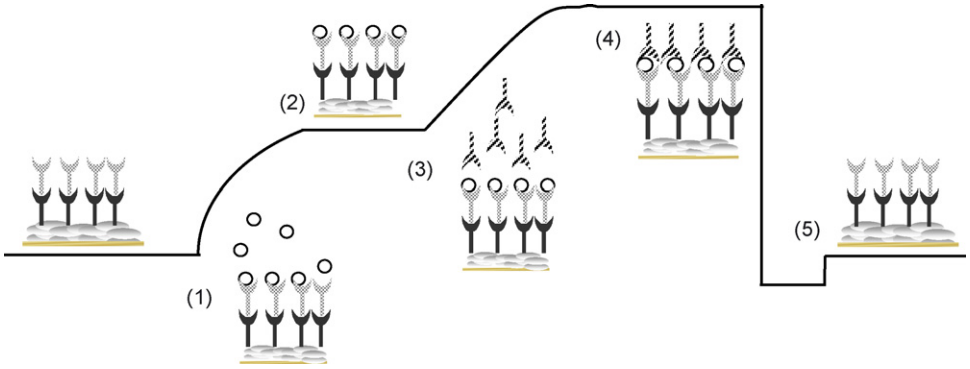

Fig. 6. Measuring assay using secondary antibodies for signal magnification. Surface with immobilised capturing Ab (anti-mouse Pab) binding the anti-vtg Mab with flowing buffer 5 μl/min (baseline), hence (1) addition of vtg; (2) washing with buffer to remove the analyte excess. Primary response recorded; thereafter (3) a secondary antibody (rabbit anti-vtg, Pab anti-vtg), specific for a different epitope of the vtg protein was added to enhance the analytical signal; (4) washing of the surface to remove the excess of secondary Pab; secondary response recorded; (5) surface regeneration by dissociation of the immunochemical reaction and return to the baseline.

4. Conclusions and future work

In the present study an optical immunosensor was developed for the rapid screening of vtg in plasma from carp (*C. carpio*). The sensor was assembled linking the analyte specific antibodies using capturing antibodies. The two types of antibodies were cross-linked which significantly improved the method as it allowed the reuse of the sensor surface. The sensor was able to detect vtg in the ppm range both in buffer and in plasma from carp. The preparation of the sensor surface took less than 1 h and the analysis of sample and standards 20 min, hence allowing for rapid screening. The optimised sensor was used for the analysis of 17β-estradiol-treated carp. Good discrimination between 17β-estradiol and control carp could be obtained, demonstrating that the sensor could be used for the rapid screening of vtg in this sample matrix. The analysis of vtg in mucus showed that this matrix could offer interesting future applications for the non-invasive vtg detection. Future work will involve the analysis of vtg in plasma and mucus from field exposed samples.

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