Lateral flow immunoassay for bisphenol A: Development of test strips and their application for ecological monitoring

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Abstract. Lateral flow tests for rapid detection of bisphenol A, a widely present toxic contaminant, were developed. Two types of markers were used as labels in combination with a visual-results estimation (control of coloration lost in the test zone) and instrumental photometric registration (decrease of coloration intensity in comparison with sample data without analyte). A test system based on gold nanoparticles allowed for detection of bisphenol A at concentrations up to 20 ng/mL visually and 0.1 pg/mL instrumentally. The use of colored latex particles as alternate labels provided visual and instrumental detection limits of 5 ng/mL and 0.3 pg/mL, respectively. The test strips were applied for bisphenol A detection in snow samples. No matrix effects influencing the analyte were found. The tests are characterized by rapidity (10 min) and simplicity of assays, including the possibility of on-site application, and may be recommended for practical use.

1. Introduction
Bisphenol A (BPA), 2,2’-bis(4-hydroxyphenyl)propane, is a plastic monomer and plasticizer with a current production rate of 2.8–3.0 million tons per year [1]. BPA is used in the production of polycarbonate plastics and epoxy resins as components of a variety of consumer products, such as beverage containers, toys, water pipes, and dentures. It has been shown that BPA is washed out from these products during use and enters the body [2]. Until recently, despite belonging to the estrogen family, BPA was considered to be of little danger due to its low affinity for estrogen receptors and an accordingly weak direct effect on the endocrine system [3]. However, recent studies have shown that BPA can stimulate cellular responses at very low concentrations. Therefore, chronic consumption of BPA may cause metabolic changes, hormonal pathologies, and oncological diseases [2, 4-6].

In the United States, Canada, and the European Union, BPA-containing materials are prohibited for the production of baby bottles and baby food packaging [7, 8]. However, BPA is still produced in large quantities. The maximum permissible level of BPA in food products is 0.6 mg/kg in the US, China, and Canada [9, 10], and 3 mg/kg in Europe [11]. In Russia, BPA is regulated for domestic water at a limit of 0.4 mg/L (Hygienic Standard GN 2.1.5.1315-03).

BPA content in environmental objects and consumer products is mainly controlled with chromatographic methods [12, 13]. However, their implementation requires expensive equipment and qualified personnel, and analysis of the results can only be carried out in specialized laboratories at a
high cost. Therefore, broad control of BPA contamination is possible only via new high-performance, simple analytical methods suitable for use in non-laboratory conditions.

A promising approach meeting the above requirements is immunochromatographic analysis (ICA). In immunochromatography, the contact of the test strip with the sample initiates all subsequent interactions between the analyte and immunoreactants, without requiring additional reagents or operator involvement. Therefore, testing can be implemented directly at the sampling location in 5–15 minutes, and subsequent decisions may be made promptly on the basis of assay results [14]. These advantages have led to the successful commercialization and widespread use of immunochromatography in different fields. Immunochromatographic systems are presently used for medical and veterinary diagnostics and for quality-control of consumer products [15], and are being actively developed for environmental monitoring [16-18].

However, with respect to BPA, the number of such developments is very limited. Two publications by Mei and co-authors [19, 20] described the traditional and enhanced formats of immunochromatography, which use one or two types of colloidal gold conjugates, respectively. Approval of these systems has been limited to the control of contaminated tap water. The analysis described in [20] was found to be substantially more sensitive and was used to detect BPA that leached out from baby bottles.

Given the significant increase of a number of markers used in immunochromatographic systems [21], it seemed advisable to compare the two most technologically advanced types: colloidal gold and latex particles.

In addition, in the present study, the use of polyclonal antibodies in immunochromatography as a crude IgG fraction of serum was realized for the first time. The use of such compounds significantly reduces the cost of reagents used to make the test system, in some cases leading to more affine antigen-antibody interactions. For this purpose, we used indirect labeling of specific antibodies for lateral flow assay implementation. Namely, native specific antibodies were combined with anti-species antibodies labeled with colloidal gold, as described in our previous studies [22, 23]. As part of the developed test approbation for BPA control, for the first time, their effectiveness was characterized for contaminant detection in snow, a known effective matrix for assessing the general state of ecosystems and the degree of contamination [24-26].

2. Materials and Methods

2.1. Reagents and materials

Anti-BPA rabbit antibodies were obtained from Agrisera Antibodies (Vannas, Sweden). Goat anti-rabbit immunoglobulin G (GARI) was obtained from Arista Biologicals (Allentown, USA). Gold chloride, nonylphenol, N-(4-dimethylaminopropyl)-N’-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide (NHS), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), N,N’-dicyclohexylcarbodiimide (DCC), and N,N’-dimethylformamide (DMF) were from Fluka (St. Louis, MO, USA). The 4,4-bis(4-hydroxyphenyl)valeric acid (BVA), bisphenol A (BPA), non-ionic detergent Tween 20, Triton X-100 detergent, and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO, USA). Blue latex particles were from Magsphere (Pasadena, CA, USA).

CNPF-backed nitrocellulose membranes and pre-treated TYPEGBF-R7L sample pads were from Advanced Microdevices (AmbalaCantt, India). CFSP223000 adsorption pads, fiberglass macroporous CFCP203000 conjugate pads, and Amicon Ultra 100 kDa centrifugal filter units were from Millipore (Billerica, MA, USA). All solutions were prepared using purified water obtained using the Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Synthesis of BPA-protein conjugates

BVA haptens were attached to BSA or STI using the modified active ester method [27]. Following the same procedures as before, BPA haptens were first activated and then conjugated to BSA or STI (15 mg/mL) using a hapten:protein molar ratio of 7:1. According to the first method, 15 µmol of BVA
was reacted with stoichiometric amounts of NHS and DCC in 0.1 mL of DMF overnight at room temperature. After centrifugation, 0.08 mL of the clear supernatant containing the active ester was dissolved in 0.310 mL of DMF and slowly added to a solution of 15 mg of protein in 0.6 mL of 0.2 M borate buffer, pH 9. The mixture was allowed to react at room temperature for 3 h with stirring. Coating conjugates were purified by dialysis against phosphate buffer solution (PBS; 50 mM, pH 7.4).

2.3. Preparation of particle-antibody conjugates
GNPs were prepared by citrate reduction of gold salt (HAuCl₄), as described in [28]. Antibodies (anti-BPA) were immobilized on GNPs by physical adsorption, according to [28, 29]. Potassium carbonate solution (0.1 M) was added to the GNP solution (D₅₂₀ = 1.0) until it reached pH 8.5, then the antiserum was added to a concentration of 50 μg/mL (based on the total IgG content in antiserum) and GARI solution at a concentration of 8 μg/mL. The mixture was incubated for 30 min with stirring at room temperature. The BSA solution (10%) was then added to a final concentration of 0.25%.

The LP-antibody conjugate was prepared according to [20] by covalent immobilization through carboxyl groups. The molar LP:IgG ratio was 1:90 and the NHS concentration was 9.2 mM. The mixture was incubated for 2 h with stirring at room temperature. The solution was blocked with 10% BSA, and then the activators were removed by centrifugation.

2.4. Preparation of immunochromatographic test strips
Reagents were applied onto membranes using an IsoFlow dispenser (Imagene Technology, Lebanon, NH, USA). Conjugate GNP was dispensed with an optical density of 4.0 (wavelength 520 nm) and conjugate LP was dispensed with a concentration of 0.5%; the conjugate load was 32 μL per 1 cm of strip width. The test zone was formed by the BPA-STI conjugate (2.0 mg/mL) and the control zone was formed by GARI (0.25 mg/mL in PBS). Two microliters of both reactants were applied per 1 cm of strip width. All concentrations and dispensing modes were chosen based on the earlier data [30].

After the reagents were dispensed and the membrane components were assembled, the lists were cut into strips 3.5 mm wide using an Index Cutter-1 (A-Point Technologies, Gibbstown, NJ, USA). Each test strip was 75 mm in length, and packaged in laminated aluminum foil using an FR-900 mini-conveyor (Wenzhou Dingli Packing Machinery, Wenzhou, Zhejiang, China), with pre-packaged silica gel (0.5 g) as a desiccant. Splitting and packing were carried out at 20–22 °C in a special room with relative humidity of <30%.

2.5. Immunochromatographic assay and data processing
The snow samples were melted at room temperature, solid impurities were filtered out, and the obtained melted snow-water was used for the ICA. The assay was carried out at room temperature. The test strip was vertically submerged into a sample for 10 min and the assay results were recorded. Digital images of the test strips were obtained with a CanoScanLiDE 90 scanner. The color intensity of the binding zones was calculated with TotalLab v2.01 software (TotalLab, Newcastle upon Tyne, UK) as described in [22].

The equation for the calibration curves approximation (4-parametric sigmoidal fitting) was as follows:

\[ y = \frac{(A - B)}{1 + (x/C)^D} + B \]

where \( x \) is analyte concentration, \( y \) is color intensity, \( A \) is the asymptotic maximum of color intensity, \( B \) is the asymptotic minimum (background value) of color intensity, \( C \) is the inflection point of the curve in the semi-logarithmic coordinates (equal to 50% inhibition of the color intensity, IC₅₀), and \( D \) is the slope of the curve at the inflection point [31].

The instrumental limit of detection (LOD) was calculated as the concentration yielding a signal reduction of three times the standard deviation of the signal for the sample without BPA. The visual LOD was interpreted as the minimal BPA concentration that caused complete absence of coloration in the test zone (i.e. its coincidence in color with neighboring regions of the strip).
3. Results and Discussion

3.1. ELISA characteristics of immunoreactants
BVA is a structural analogue of BPA containing a carboxyl group for covalent bonding to a carrier protein. BVA haptens were attached to STI using the modified active ester method [27]. BSA was used as immunogen reactant and STI was an alternative protein carrier. Polyclonal antibodies against BPA and hapten-protein conjugates were characterized with ELISA. Figure 1(a) shows the effect of the protein carrier on interactions between antibodies and immobilized hapten-protein conjugates. The chosen optimal conditions for the ELISA assay procedure provided maximum sensitivity and accuracy. BPA and BVA were used as free antigens. ELISA based on antibodies against BPA is characterized by a working range of 0.3–20 μg/mL with a detection limit of 0.15 μg/mL and an IC₅₀ (concentration causing 50% inhibition of binding) of 2.4 μg/mL (Figure 1(b)).

The polyclonal antibodies used in the study were characterized by cross-reactivities with respect to BPA and BVA (Figure 1(b)). The antibodies are more affine to BVA. However, the reactivity of the antibodies to BPA was sufficient for their use in the development of rapid test systems.

![Figure 1](image1.png)

**Figure 1.** Characteristics of polyclonal antibodies against BPA and BVA-STI conjugate by ELISA. (a) Titration of antibodies using BVA-STI conjugate; (b) competitive curves for BPA (1) and BVA (2).

3.2. Development and characterization of immunochromatographic system based on GNP
First, a standard immunochromatographic system based on GNP using polyclonal antibodies as a capture reactant for determining BPA was developed. The principle behind BPA determination by means of an immunochromatographic test system is the competitive interaction of the antibodies labeled by GNP with the free antigen in the sample and with the hapten-protein conjugate immobilized in the test zone. The following parameters were varied for identification of the optimal completion of the test system: (i) concentration of polyclonal antibodies in the GNP-antibody conjugate; (ii) concentration of the conjugate BVA-STI applied in the test zone; (iii) quantity of the detecting conjugate between GNP and specific antibodies; and (iv) composition of the reaction medium. Based on earlier experiments, an optimum working nitrocellulose membrane with a pore size of 8 μm allows for testing non-viscid samples [29, 32].

The polyclonal antibody concentration during the conjugate synthesis ranged from 5 to 20 μg/mL, with an optimal concentration of 10 μg/mL. Smaller concentrations did not provide the necessary conjugate stability, and the sensitivity of the assay did not significantly differ with larger concentrations.

The concentration of the BVA-STI conjugate in the test zone was varied from 0.2 to 2.0 mg/mL (Figure 2). According to the data obtained, at concentrations of immobilized BVA-STI conjugate of
≤0.5 mg/mL, the staining intensity was low, leading to unreliable results. Concentrations of 1 mg/mL and 2 mg/mL yielded comparable results. The optimal concentration of the BVA-STI conjugate immobilized in the test zone was found to be 1 mg/mL. This provided maximum color intensities and a minimum of relative errors (<12.5%).

![Graph](image)

**Figure 2.** Dependence of the coloration intensity of the test zone on the concentration of BPA in the sample for different concentrations of the immobilized BVA-STI conjugate: (1) 2 mg/mL, (2) 1 mg/mL, and (3) 0.5 mg/mL.

Using the chosen concentrations of reactants, the calibration curve shown in Figure 3 was obtained. The developed immunochromatographic assay for BPA was characterized by detectable concentrations in the range of 0.001–20 ng/mL with an IC₅₀ of 0.025 ng/mL. The colorimetric detection limit was 0.1 pg/mL, and the visual detection limit was 20 ng/mL.

![Graph](image)

(a) (b)

**Figure 3.** Test strips (a) and competitive curves for BPA detection based on GNPs.
This colorimetric detection limit corresponds to the established standards. However, for visual detection of results, a more sensitive test system is required, so we characterized alternative markers in further studies.

Since BPA refers to substances used to make plastic containers, detergents may also be present in samples together with BPA. Thus, the tested sample may contain surface active compounds, such as Triton X-100. This compound also adversely affects human health, and evaluation of its presence should be carried out individually, excluding cross-reactivity with BPA. Moreover, detergent is a necessary compound in immunoassays for the exclusion of non-specific interactions. Thus, it should be added to the ICA test, but should not cause cross-reactivities.

The competitive interactions between the anti-BPA antibodies and Triton X-100 were characterized by varying the concentration of Triton X-100 from 0 to 5% (w/v). It was shown that the detergent did not influence BPA detection. For further work, Triton X-100 detergent was used at a concentration of 0.3%, as commonly recommended [33].

3.3. Development and characterization of an immunochromatographic system based on LPs

One method of reducing the detection limit is based on replacing the colorimetric marker. Some successful results in this direction are described in the literature. For example, the replacement of GNPs by fluorescent markers reduces the detection limit by more than 20 times [34]. The use of carbon nanotubes can reduce the detection limit by 50 times due to the greater contrast of this label [35, 36]. In our work, latex particles (LPs) were selected as alternative markers. LPs are characterized by a large size and the concentration of a large number of dye molecules per particle. In addition, colored LPs, which are inexpensive and mass-produced, have great practical potential and are not characterized in comparative terms.

For the present study, LP-antibody conjugates were synthesized. The chosen antibody concentration was 167 µg/mL, providing a minimum detection limit and forming a high coloration intensity of the test zone (Figure 4).

![Figure 4](image_url)

**Figure 4.** Dependence of the staining intensity of the test zone on the concentration of antibodies in the conjugate synthesis with LPs.

The pH of the buffer solutions for conjugate synthesis varied between 4.0 and 9.0. The final synthesis of the conjugate and its storage were carried out in carbonate buffer, pH 9.0. This medium ensured the stability of the obtained preparation.

The following parameters were varied for selection of the optimal completion of the test system: (i) concentration of the conjugate BVA-STI applied in the test zone; (ii) quantity of the detecting
conjugate LP-antibodies; and (iii) composition of the reaction medium. Using the chosen concentrations of reactants, the calibration curve shown in Figure 5 was obtained.

![Test strips and calibration curve](image)

**Figure 5.** Test strips (a) and competitive curves for BPA detection based on LPs.

The immunochromatographic assay for BPA was characterized by detectable concentrations in the range of 0.001–5 ng/mL. The colorimetric detection limit was 0.3 pg/mL, and the visual detection limit was 5 ng/mL. Thus, blue LPs allow reduction of the detection limit by 4 times in comparison with the GNPs-based test system.

### 3.4. Approbation of developed test system

The developed test system was used to determine BPA levels in snow (Figure 6). It was shown that there were no differences between the calibration curves obtained in a snow sample and a buffer solution. The GNPs-based and LP-based systems demonstrated similar tolerances to the snow matrix.

![Calibration curves](image)

**Figure 6.** Calibration curves for BPA detection in PBST and snow (as an example for GNPs).

The main advantage of the developed test system for determining BPA levels in snow samples was the absence of the need for sample preparation.
4. Conclusion
For the evaluation of BPA levels, the best results were obtained using latex particles as labels. This is substantially due to the large size of these particles compared to gold nanoparticles, which are the traditional immunochromatographic labels. The established patterns can be used for the development of rapid, highly sensitive test systems for the detection of compounds of different classes.

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