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Microplate immunoassay technique using polyelectrolyte carriers: kinetic studies and application to detection of the herbicide atrazine

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Abstract

Electrostatic interaction between water-soluble polyelectrolytes (poly(methacrylate) polyanion and poly(*N*-ethyl-4-vinylpyridinium) polycation) was characterized as an approach for the acceleration of microplate enzyme immunoassays (ELISAs). The proposed assay technique includes (1) carrying out of the immune interactions in the solution that contains a tracer antigen, antigen–peroxidase conjugate, antibodies, and polyanion–protein A conjugate, (2) binding of the polyanion-containing complexes formed with the polycation that was immobilized in microplate wells, (3) washing of the wells, and (4) optical measurement of peroxidase activity using a substrate solution. Kinetic and concentration dependencies of the assay were studied for the herbicide simazine as a model antigen. It was shown that the quantity of the interpolymeric complexes formed increased significantly within a narrow range of polyanion/polycation ratio. Either polycation alone or polycation conjugated to soybean trypsin inhibitor could be used as immobilized reactants; both variants of the assay having the same sensitivity. With the polyelectrolyte ELISA assay, immune complexes were detected in two short steps of 8 and 5 min, whereas the traditional ELISA format required 60 min incubations to saturate binding sites of the immobilized antibodies. After optimization, the polyelectrolyte ELISA was utilized for detection of the herbicide atrazine. The sensitivity of this assay was 0.03 ng/ml, with a total duration of 40 min. The coefficient of variance of the assay ($n = 4$) was 1.4–8.0% for atrazine concentrations in the range 0.05–1 ng/ml.

Keywords: Immunoassay; ELISA; Polyelectrolytes; Herbicides; Atrazine; Simazine

1. Introduction

One of the most important problems in the development of immunoassay techniques consists of combining high sensitivity and speed [1,2]. Enzyme-linked immunosorbent assay (ELISA) and many other traditional immunoassay formats need relatively prolonged incubations to reach chemical equilibrium of the immune interactions. The extremely dilute reagents used in highly sensitive assays exacerbate this problem. The rate of reaction is governed by diffusion limitations to the binding between immobilized reactants and reagents in solution [3]. Therefore, techniques that carry out the immune interaction in solution with the subsequent quick heterogeneous separation of the reactants are of significant interest currently.

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To solve this problem, several approaches have been proposed — immobilization of reactants on finely ground suspended particles or on carriers with temperature-dependent solubility, precipitation of immune complexes by polyethylene glycol, etc. [4–6]. However, such assays are practical only in combination with additional devices for separation of the insoluble phase. Therefore, the range of their application is limited.

Previously [7], we have proposed interaction between poly(*N*-ethyl-4-vinylpyridinium) polycation and poly(methacrylate) polyanion as an approach for immunoassay acceleration. These linear water-soluble polyelectrolytes have been used as carriers for immunoreactants. High densities of the oppositely charged groups in their molecules ensure an extremely high rate and affinity of the polycation–polyanion interaction. By virtue of this property, the immune complexes can be rapidly separated at any time during the assay. This approach was illustrated using a modified ELISA scheme for detection of the herbicide simazine based on immobilization of the polycation in microtiter plate wells [8,9].

The present work is devoted to optimization of the assay regime and application of the resulting information for detection of another herbicide, atrazine. The choice of simazine (2-chloro-4,6-di-(*N*-ethylamino)-1, 3,5-triazine) and atrazine (2-chloro-4-(*N*-ethylamino)-6-(*N*-isopropylamino)-1,3,5-triazine) as model antigens was based on the high demand for their detection for environmental monitoring and food quality control [10–13].

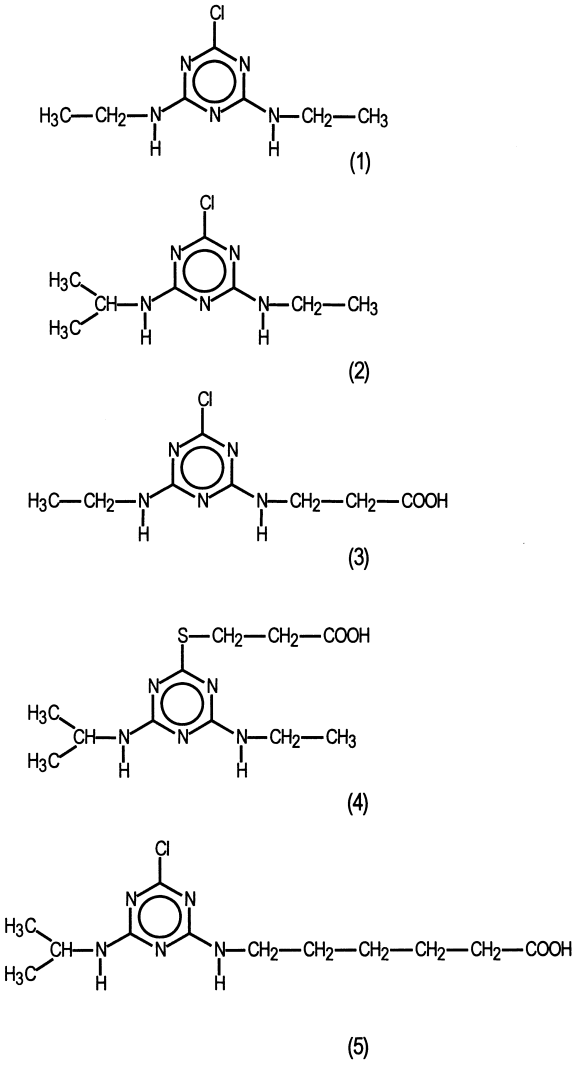

Fig. 1. Structural formulae of simazine (1), atrazine (2) and their carboxylic derivatives that were used for conjugation with proteins (3–5).

2. Experimental

2.1. Materials

Riedel De Haen preparations of simazine and atrazine were used as standard tracer antigens for the immunoassays. The activated pesticide derivatives (see Fig. 1) were synthesized according to [14] and generously provided by Dr. S.A. Eremin, Moscow State University.

Hemocyanin from *Paralithodes camtschatica* (HC, generously provided by Dr. I.Yu. Sakharov, Moscow State University), bovine serum albumin (BSA, Reanal, Hungary), soybean trypsin inhibitor (STI, Reanal) and horseradish peroxidase (HRP, $RZ = A_{403}/A_{280} = 3.0$, Biolar, Latvia) were used for syntheses of protein–pesticide conjugates.

Protein A from *Staphylococcus aureus* (Vostok, Russia), *N*-hydroxysuccinimide (Sigma), 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide (CalBiochem), cyanuric chloride (Fluka), dimethylformamide, Triton X-100 (both from Serva), and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Boehringer Mannheim) were also used in

the experiments. Constituents of buffer solutions and other chemicals were of analytical grade.

ELISA was carried out in Costar optically transparent polystyrene microplates.

2.2. Preparation of water-soluble polyelectrolytes

Poly(methacrylic) acid (PMA) and poly(4-vinylpyridine) (PVP) were synthesized and fractionated according to previously described techniques [15,16]. PMA was prepared by radical polymerization of methacrylic acid (Fluka) in benzene at 60°C in nitrogen atmosphere using 2,2′-azobisisobutironitrile as the polymerization initiator. Benzene is a cancer suspect agent and must be used with caution. PVP was prepared by radical polymerization of vinylpyridine (Fluka) in methanol at 60°C under a nitrogen atmosphere using 2,2′-azobisisobutyronitrile as the polymerization initiator. Samples of PMA and PVP were fractionated with ethyl acetate-methanol. Weight-average molecular weight, M_w, measured by light-scattering, was determined in methanol at 25°C. A narrow fraction of PMA with an average molecular mass of 260 kDa was used. Differences of PMA molecules by polymerization degree were characterized by the value $M_w/M_n = 1.2$, where M_n was a number average molecular mass [7]. Fractionated preparations of PVP were quaternized exhaustively by ethyl bromide in order to prepare poly(*N*-ethyl-4-vinylpyridinium) bromide (PEVP). The quaternization degree was more than 90% according to spectral data [7]. The PEVP preparations with $M_w = 1740$, 2900, and 3900 kDa ($M_w/M_n = 1.3$ for all them) were used. In addition, another PEVP preparation was obtained from PVP by a separate technique [17]. This method differed from the above techniques in that hydroxyl groups were introduced into some of the ethyl residues. The modified ethyl residues accounted for 1% of the total ethyl groups in the polymer. The preparation of modified PEVP obtained had an M_w equal to 9800 kDa and M_w/M_n equal to 1.3.

2.3. Obtaining antibodies

To raise anti-simazine antibodies, Chinchilla rabbits weighing 3–4 kg were immunized according to the following procedure [18]. An immunogen (simazine–BSA or simazine–HC) dissolved in 0.05 M K-phosphate buffer, pH 7.4, with 0.1 M NaCl (hereafter named PBS) was emulsified with an equal volume of Freund's complete adjuvant (Difco) to a final concentration of 0.5 mg/ml (by protein). For the first immunization, 1.0 ml of this mixture was injected four times (Days 1, 15, 29 and 43) intradermally on multiple sites on the back from scapula to sacrum. After 2 months (Day 103), the first boost was carried out: the rabbits were injected intravenously and intradermally with 0.3 and 0.2 ml of the immunogen, respectively, dissolved in PBS at the same concentration. 7 days later (Day 110), the rabbits were bled. This procedure was repeated on a monthly basis (boosting: Days 133, 163 and 193, bleeding: Days 140, 170 and 200).

Rabbit antisera against atrazine were obtained as described in [19]. Antisera were extracted by settling blood samples for 16 h at +4°C. Upper layers were carefully collected, divided into aliquots and stored at −20°C.

2.4. Syntheses of herbicide–protein conjugates

The following procedure was used [18,20]. The simazine (or atrazine) carboxylate derivative (0.05 mmol) was diluted in 0.5 ml of dimethylformamide, then 0.1 mmol *N*-hydroxysuccinimide and 0.1 mmol 1-ethyl-3(3-dimethylaminopropyl)carbodiimide were added, and the mixture was stirred for 2 h at room temperature. Then, the solution of the activated hapten was cooled to +4°C and added to the cooled protein solution (4 mg BSA/2 mg HC/4 mg STI/4 mg HRP) in 0.5 ml of 0.02 M Na-carbonate buffer, pH 9.5, containing the same volume of dimethylformamide. The initial hapten/protein molar ratio was varied from 3 : 1 to 50 : 1 (for the preparation of tracer conjugates for immunoassays) and up to 500 : 1 (for immunogens). The resultant mixture was incubated with stirring for 1 h at room temperature and for 16 h at +4°C. The resulting conjugates were separated from low molecular weight compounds in the reaction mixture by gel-filtration on Sephadex G-25 (Pharmacia, 1 cm × 20 cm column, in PBS) and/or by dialysis.

2.5. Conjugation of PEVP with STI

s-Cyanuric chloride (3.9 mg) dissolved in 0.4 ml of 50% dioxane was added dropwise under stirring to

1 ml of a water solution of the hydroxy-containing PEVP preparation (6 mg/ml). After this, 0.5 mg of STI dissolved in 0.1 ml of 0.1 M K-phosphate buffer, pH 7.8, was added, and the mixture was stirred for 10 h at room temperature. The conjugate obtained was separated from low molecular weight compounds in the reaction mixture by dialysis.

2.6. Conjugation of PMA with protein A

1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (6 mg) was added to 1 ml of PMA sodium salt solution (6 mg/ml), and the pH was adjusted from 5.0 to 7.7–7.8 by addition of 1 M KOH. The mixture was incubated for 5 min under vigorous stirring at room temperature, then a solution of 6 mg of *N*-hydroxysuccinimide and 6 mg of protein A in 1 ml of 0.1 M K-phosphate buffer, pH 7.8, was added. The mixture was incubated for 2 h at constant pH and room temperature. The reaction products were separated from low molecular weight compounds by gel-filtration on Toyopearl HW-55 (Toyosoda, 1.6 cm × 100 cm column) in PBS and/or by dialysis.

2.7. Precipitation of PMA and PEVP in solution

Solutions of PMA and PEVP (M_w = 2900 kDa) in PBS were mixed in the wells of microplates (0.1 ml + 0.1 ml). The final concentration used for PEVP varied from 40 to 600 ug/ml, and for PMA, from 10 to 1250 ug/ml. The resulting mixture of PEVP and PMA was incubated for 15 min at room temperature with stirring. For kinetic studies, the duration of the incubation was varied from 1 to 60 min. The turbidity of the interpolyelectrolyte complexes formed in the mixture was measured with a Multiskan EX vertical photometer (Labsystems) at the minimum available wavelength of 405 nm.

2.8. Polyelectrolyte ELISA

Solution of PEVP or the PEVP–STI conjugate (100 ul, 5 ug/ml by PEVP, in PBS) was added into each microplate well and incubated for 16 h at +4°C (or for 2 h at 37°C). The wells were washed with PBST (PBS, containing 0.05% Triton X-100). Four solutions in PBST were successively added into a second incubation microplate: herbicide (25 ul, row of dilutions from 3 pg/ml to 100 ng/ml), herbicide–HRP conjugate (25 ul, 4 ug/ml by HRP), anti-herbicide antiserum (50 ul, dilution 1 : 1000), and protein A–PMA conjugate (50 ul, 80 ug/ml by protein A). The resulting mixture was incubated for 5 min at room temperature with orbital shaking. Then aliquots of the mixture (100 ul) were put into the PEVP-treated plate and incubated for 8 min at room temperature. For kinetic studies, the duration of these steps was varied from 1 to 30 min. The wells were washed, and peroxidase activity was measured as described in Section 2.10.

2.9. Traditional ELISA

Protein A solution (100 ul, 2.5 ug/ml) in PBS was added into wells of microtiter plates and incubated for 1.5 h at 37°C (or for 16 h at 4°C). Subsequently, the wells were washed four times with PBST. The specific antiserum was added (100 ul, dilution 1 : 1000 in PBST), incubated for 1 h at 37°C, and the microplate was washed repeatedly. Then, 50 ul of herbicide solutions (row of dilutions from 3 pg/ml to 100 ng/ml, in PBST) and 50 ul of the herbicide–HRP conjugate (1 ug/ml by HRP, in PBST) were added into the wells. The microplate was incubated for 1 h at 37°C and then washed. For kinetic studies of this step, its duration was varied from 5 min to 3 h. Finally, peroxidase activity for immune complexes formed on the surface of the wells was measured as described in Section 2.10.

For kinetic studies of the protein A–antibody binding, peroxidase-labeled antibodies were used. These peroxidase conjugates were synthesized using the traditional periodate technique [21] and purified by gel-filtration on Toyopearl HW-55 (Toyosoda, 1.6 cm × 100 cm column) in PBS.

2.10. Peroxidase activity measurement

The substrate solution was prepared using ABTS (0.7 mM) and H_2O_2 (1.8 mM) in 30 mM Na-acetate buffer, pH 4.5. This solution (100 ul) was added into each microplate well and incubated for 10–30 min at room temperature. The optical density of the product was measured with a Multiskan EX vertical photometer (Labsystems) at 405 nm.

3. Results and discussion

3.1. Kinetic dependencies for traditional and polyelectrolyte ELISA

As mentioned above, the interaction between immobilized and dissolved reactants represents a rate limiting stage in performing ELISA techniques. Therefore, we compared the influence of the duration of the heterogeneous reaction on the assay parameters of both the traditional and the polyelectrolyte ELISA. A competitive format with immobilized antibodies and labeled antigen was used in both ELISA techniques compared here. Earlier, we have described the increase in sensitivity of a pesticide ELISA after the immobilization of antibodies through their interaction with adsorbed staphylococcal protein A [18]. This phenomenon may be explained as a result of more preferable orientation of the antibodies after the binding of their Fc-regions. So the developed and standard ELISA techniques were based on the measurements of protein A–antibody–pesticide–peroxidase complexes formed on the microplate surface. The resulting color development is directly proportional to the complexes formed on the microplate surface.

Kinetic curves for the interaction between immobilized protein A and dissolved antibodies are given in Fig. 2. Saturation of the binding sites is reached after about 60 min of incubation, and the shortening of this stage leads to a significant decrease in the measured signals. The binding of the dissolved simazine–peroxidase conjugate with the previously formed immobilized protein A–antibody complexes was characterized by similar kinetics: full binding is reached after 90 min of incubation, and even 60 min of incubation ensures only 70% saturation.

The polyelectrolyte ELISA includes two steps in the formation of the detected complex — immune interaction in solution and polycation–polyanion binding of the solid phase surface. The heterogeneous polyelectrolyte reaction is significantly more rapid as compared to the heterogeneous immune reaction outlined above. As can be seen from Fig. 3, equilibrium of the polyelectrolyte binding is reached in 8 min. This difference in speed is a result of two factors: (1) high surface density of charged groups that are able to interact, and (2) high cooperation in the formation of additional

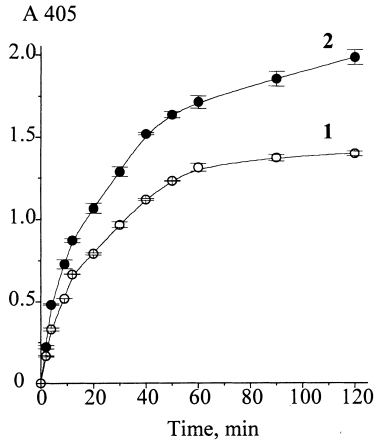

Fig. 2. Kinetics of protein A–antibody interaction on the microplate surface. Protein A concentration during adsorption – 3 ug/ml. Curves 1–2 correspond to concentrations of peroxidase-labeled antibodies of 1.3 and 2.0 ug/ml, respectively. Duration of the peroxidase enzymatic reaction is 10 min. On *X*-axis – time of the interaction, on *Y*-axis – optical density of ABTS oxidation products. Measurements were carried out in triplicate.

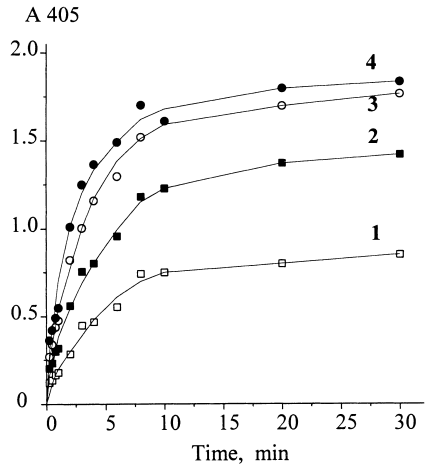

Fig. 3. Kinetics of polycation–polyanion interaction on the microplate surface. PEVP (M_w = 3900 kDa) concentration during adsorption was equal to 5 ug/ml. Curves 1–4 correspond to PMA–protein A concentrations of 0.1, 0.2, 0.4, and 0.8 ug/ml (by PMA), respectively. The complexes formed were detected following a 1 h incubation with anti-simazine antisera (dilution 1 : 1500) and the simazine–peroxidase conjugate (4 ug/ml by peroxidase). On *X*-axis – time of the PEVP–PMA interaction, on *Y*-axis – optical density of ABTS oxidation products. Duration of the peroxidase reaction was 30 min.

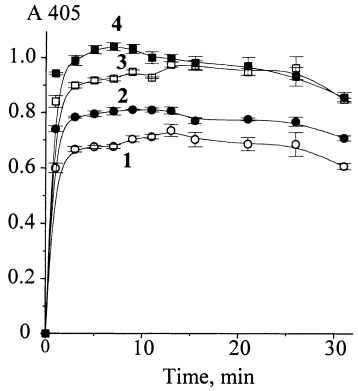

Fig. 4. Kinetics of the formation of immune complexes in solution during the proposed polyelectrolyte ELISA. The reaction mixture contained 4 ug/ml of the conjugate polyanion–protein A (by protein A), anti-simazine antisera (dilution 1 : 1500) and the simazine–peroxidase conjugate. Curves 1,2 correspond to concentration of the simazine–peroxidase conjugate 1 ug/ml (by peroxidase), and curves 3,4 – to the concentration 3 ug/ml. The incubation of the reaction mixture was followed by the reaction with immobilized polycation (M_w = 3900 kDa) during 5 min (curves 1,3) or 20 min (curves 2,4). Concentration of the polyanion during adsorption was 5 ug/ml. On *X*-axis – time of the interactions in solution, on *Y*-axis – optical density of ABTS oxidation products. Duration of the peroxidase reaction was 10 min. Measurements were carried out in triplicate.

electrostatic bonds between the polymer molecules. Replacement of the immune reactions in solution also leads to their acceleration [4]. We have studied the influence of the duration of this step by the ELISA technique that included an additional incubation of the reactants' mixture with the immobilized polycation. This technique is not specific for each individual kinetic reaction (antibody–antigen, antibody-labeled antigen, protein A–antibody), but it gives adequate information for the choice of assay duration. The kinetics given in Fig. 4 shows the two regimes of the following heterogeneous separation, 20 and 5 min. As we have shown above, a 20 min incubation ensures full saturation of the polycation binding sites, whereas after a 5 min incubation, the polycation–polyanion reaction has not quite reached equilibrium. Nonetheless, kinetic dependencies of the immune reactions were similar enough to conclude that the 5 min incubation is sufficient for reaching a maximal quantity of the detected complexes.

We conclude that the proposed approach allows us to eliminate the prolonged incubation steps of heterogeneous immune recognition from the ELISA procedures and to replace the technique with two short steps to detect the complex formation.

3.2. Influence of polycation and polyanion concentrations on the formation of the detected complexes

As the polyelectrolyte molecule can interact simultaneously with several molecules of the oppositely charged polymer, the composition of the complexes formed depends significantly on the size of the polymers and the polycation/polyanion ratio. These effects can be shown directly by studying the turbidity of the solutions obtained after mixing of the polycation and the polyanion at different concentrations. Fig. 5 demonstrates the typical concentration dependencies for such experiments. Kinetic studies demonstrated that, in all cases, the turbidity reached a stable level after 2–3 min of incubation. So the curves shown in Fig. 5 represent data collected after an equilibrium was reached. As can be seen, each concentration of the polycation corresponds to a relatively narrow interval of polyanion concentrations that provide maximum formation of large-size aggregates of the polymers. The interaction of adsorbed polycation with the dissolved polyanion also leads to formation of the complexes with different composition and affinity. In

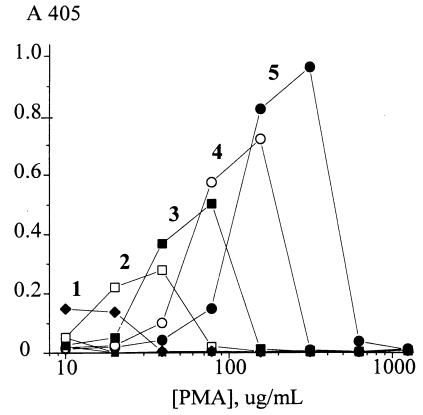

Fig. 5. Aggregation of the polycation and the polyanion in solution for different polycation/polyanion ratios. Curves 1–5 correspond to PEVP concentrations of 37.5, 75, 150, 300, and 600 ug/ml, respectively. On *X*-axis – concentration of the PMA–protein A conjugate, on *Y*-axis – turbidity of solution after 15 min of incubation.

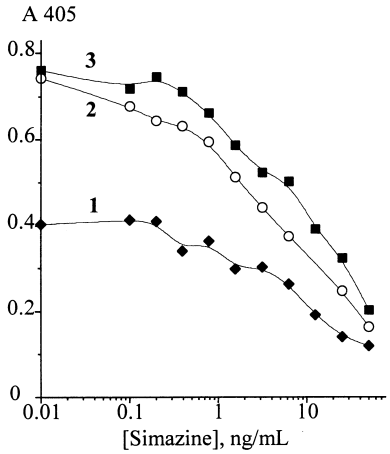

Fig. 6. Choice of optimal polycation preparation for adsorption on microplate wells. Curve 1 corresponds to PEVP with M_w = 2900 kDa, curve 2 – to polycation with M_w = 3900 kDa, curve 3 – to the conjugate of polycation with M_w = 9800 kDa with soybean trypsin inhibitor. Polycation preparations were adsorbed from a concentration of 5 ug/ml (by PEVP). Concentration of the polyanion–protein A conjugate – 2 ug/ml (by protein A), dilution of anti-simazine antisera – 1 : 1500, concentration of the simazine–peroxidase conjugate – 1.2 ug/ml (by peroxidase). On *X*-axis – concentration of simazine, on *Y*-axis – optical density of ABTS oxidation products. Duration of the immunochemical stage – 20 min, duration of the peroxidase reaction – 30 min.

[9], we have shown that this heterogeneous interaction was also characterized by a significant increase in the quantity of the detected complexes formed, within a narrow range of the polycation/polyanion ratio. So, for the developing polyelectrolyte ELISA, we have chosen the polycation concentration that ensured saturation of the polystyrene adsorption sites, and the polyanion concentration that resulted in maximal binding of the enzyme label for the predetermined concentration of the polycation. We have found that the necessary concentration of the polycation was equal to 5 ug/ml, and the concentration of the polyanion–protein A conjugate was 80 ug/ml (by polyanion).

We have compared properties of the PEVP molecules differing in their average molecular weight. Fig. 6 shows the curves of competitive simazine detection that were obtained for the different preparations. The adsorbed polycation with M_w = 1740 kDa was characterized by an extremely low level of polyanion binding. Thus, with this reagent, the difference between the non-specifically bound and specifically bound complexes was not reliable. Probably, this phenomenon is associated with the lack of ability of these relatively short molecules to form durable complexes with the solid-phase and polyanion molecules simultaneously. The polycation with M_w = 3900 kDa demonstrates significantly higher binding as compared to the preparation having M_w = 2900 kDa (see curves 1 and 2); this fact is in accordance with the above interpretation. The PEVP with M_w = 9800 kDa formed an unstable solution in PBS. Development and use of the calibration curve for this polycation was difficult because of the high variability of the experimental points. To solve this problem, we have conjugated this polycation with a protein carrier, soybean trypsin inhibitor, in order to increase the solubility and ability for adsorption. The resulting preparation could not be distinguished in ELISA parameters from the best non-modified PEVP preparation (see curves 2 and 3). Both variants — PEVP with M_w = 3900 kDa and the conjugate of soybean trypsin inhibitor with PEVP having M_w = 9800 kDa — may be recommended for further development of ELISA.

3.3. Choice of optimal concentrations of specific reactants for atrazine detection

The proposed ELISA format is connected with the application of both universal reactants (polycation and the polyanion–protein A conjugate) that can be used in the detection of any antigen, and specific reactants (antibodies and the antigen–peroxidase conjugate) that ensure the detection of a strictly specified compound or closely related compounds. After optimizing the concentration of non-specific reactants as described above, we optimized the concentrations of the specific reagents for detection of the pesticide atrazine.

A comparison of two carboxylic derivatives of atrazine as haptens for the preparation of the atrazine–peroxidase conjugate is presented in Fig. 7. Atrazine did not compete with the conjugate of derivative (5) in checkerboard titrations, and we have used only the conjugate of derivative (4) in the following experiments.

Determination of the optimal dilution of antisera and atrazine–peroxidase concentration was based on the experimental data that are given in Figs. 8 and 9. We have chosen the values of both parameters that

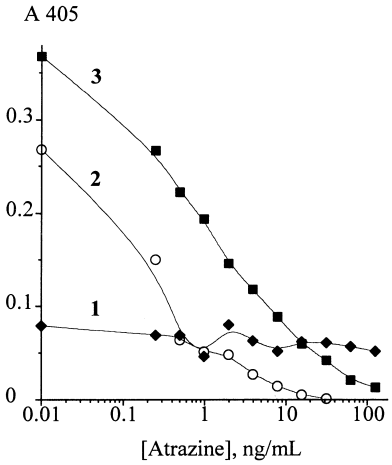

Fig. 7. Comparison of different atrazine–peroxidase conjugates by the traditional ELISA technique. Curve 1 corresponds to the peroxidase conjugate with derivative (5) of atrazine, curves 2, 3 – to two lots of the peroxidase conjugate with derivative (4) of atrazine. The assay regime is described in Section 2.9. On *X*-axis – concentration of atrazine, on *Y*-axis – optical density of ABTS oxidation products. Duration of the peroxidase reaction – 20 min.

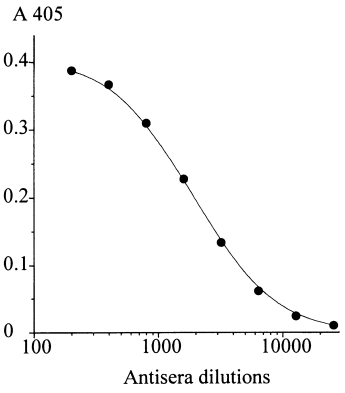

Fig. 8. Influence of antisera dilution on the formation of the polycation–polyanion–protein A–antibody–antigen–peroxidase complexes during the polyelectrolyte ELISA. Concentration of the atrazine–peroxidase conjugate – 4 ug/ml (by peroxidase). On *X*-axis – antisera dilutions, on *Y*-axis – optical density of ABTS oxidation products. Duration of the peroxidase reaction – 20 min.

correspond to a high amplitude of detected signal, low level of non-specific label binding and maximal sensitivity of competitive atrazine detection. In connection with the latter reason, the concentrations chosen were smaller than the saturation level of specific label binding. On account of this approach, there is no

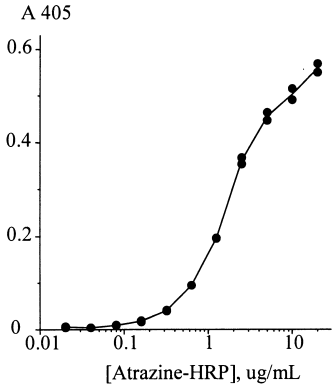

Fig. 9. Influence of the concentration of the atrazine–peroxidase conjugate on the formation of the polycation–polyanion–protein A–antibody–antigen–peroxidase complexes during the polyelectrolyte ELISA. Antisera dilution – 1 : 1000. On *X*-axis – concentration of the atrazine–peroxidase conjugate, on *Y*-axis – optical density of ABTS oxidation products. Duration of the peroxidase reaction – 20 min.

abundance of antibodies or conjugate in the reaction mixture that will create obstacles in detection of the competitor (free atrazine molecules). The regime optimized here for the polyelectrolyte ELISA of atrazine is given in Section 2.

3.4. Characteristics of the atrazine immunoassay

Finally, we have compared the polyelectrolyte ELISA format developed herein with the traditional competitive assay of atrazine based on the same reactants. The calibration curves for these optimized assays are given in Figs. 10 and 11. As can be seen, the proposed assay compares well with the traditional one in sensitivity. The limit of atrazine detection by the polyelectrolyte ELISA technique is 0.03 ng/ml. At the same time, the polyelectrolyte technique is significantly faster. Its total duration is 30 min, while for the traditional assay, this parameter is equal to 1.5 h (if the interaction between protein A and antibodies is interpreted as a preliminary stage that is carried out before the analysis of atrazine-containing samples).

The proposed assay was characterized by high reproducibility. Coefficients of variance for four parallel tests using different atrazine concentrations are given in Table 1. The coefficients of variance range from 1.4 to 8.0%, as is expected of typical ELISA tests.

Table 1
Coefficients of variance ($n = 4$) on the detection of various concentrations of atrazine by the polyelectrolyte ELISA

Atrazine concentration (ng/ml)	Coefficient of variance	
	Immobilization of PEVP with $M_w = 3900$ kDa (%)	Immobilization of PEVP with $M_w = 9800$ kDa conjugated with soybean trypsin inhibitor (%)
1.0	5.0	8.0
0.3	4.2	2.1
0.1	3.6	2.3
0.05	2.9	1.4

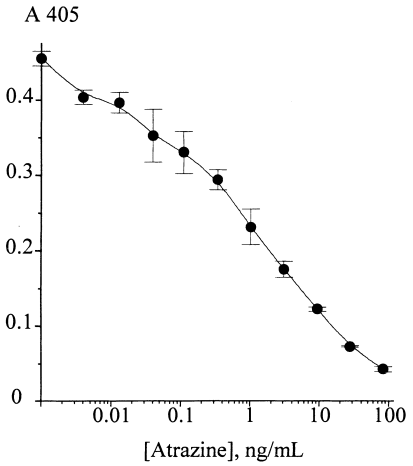

Fig. 10. Calibration curve for atrazine detection by the traditional ELISA. Concentration of reactants and duration of steps are given in Section 2.9. On *X*-axis – atrazine concentration in the tested samples, on *Y*-axis – optical density of ABTS oxidation products. Duration of the peroxidase reaction – 20 min. Measurements were carried out in four replicates.

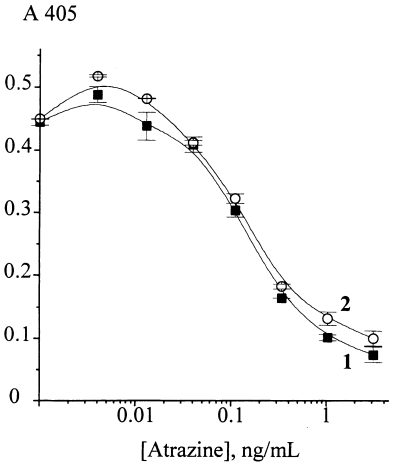

Fig. 11. Calibration curves for atrazine detection by the developed polyelectrolyte ELISA technique described here. Curve 1 corresponds to the immobilization of PEVP with $M_w = 3900$ kDa, curve 2 – to the immobilization of PEVP with $M_w = 9800$ kDa conjugated with soybean trypsin inhibitor. Concentration of reactants and duration of steps are given in Section 2.8. On *X*-axis – atrazine concentration in the tested samples, on *Y*-axis – optical density of ABTS oxidation products. Duration of the peroxidase reaction – 20 min. Measurements were carried out in four replicates.

4. Conclusions

The proposed polyelectrolyte technique permits a significant increase in the speed of the resulting ELISA without loss of sensitivity. By using the polyanion–protein A conjugate, the described assay does not require the preparation and the purification of specific antibody derivatives. Therefore, the same scheme can be easily adapted for other analytes.

The sensitivity of the polyelectrolyte and standard ELISAs described here are commiserate with other reports on the immunodetection of atrazine [14,19,20,22]. The sensitivity is adequate for the environmental detection of atrazine as expected by many countries. This modification of the ELISA technique may be recommended for practical application in agricultural and ecological monitoring and be more generally applicable in food and clinical chemistry.

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References

[1] D. Wild (Ed.), The Immunoassay Handbook, Stockton Press, New York, 1994.
[2] M.-P. Marco, S.J. Gee, B.D. Hammock, Trends Anal. Chem. 14 (1995) 415.
[3] E. Sciutto, B. Garat, A. Ortega, C. Larralde, Mol. Immunol. 24 (1987) 577.
[4] T. Ngo, H.M. Lenhoff, Enzyme-Mediated Immunoassay, Plenum Press, New York, 1985.
[5] T.C. Gribnau, J.H. Leuvering, H. Van Hell, J. Chromatogr. 376 (1986) 175.
[6] N. Monji, A.S. Hoffman, Appl. Biochem. Biotechnol. 14 (1987) 107.
[7] B.B. Dzantiev, M.J. Choi, J. Park, J. Choi, O.G. Romanenko, A.V. Zherdev, S.A. Eremin, V.A. Izumrudov, Immunol. Lett. 41 (1994) 205.
[8] B.B. Dzantiev, A.V. Zherdev, E.V. Yazynina, V.A. Izumrudov, in: Abstr. of the IXth IUPAC Pesticide Congress, London, 2–7 August 1998, 7A-055.
[9] E.V. Yazynina, A.V. Zherdev, B.B. Dzantiev, V.A. Izumrudov, S.J. Gee, B.D. Hammock, Anal. Chem., in press.
[10] K.S. Nam, J.W. King, J. Agric. Food Chem. 42 (1994) 1469.
[11] D. Knopp, Anal. Chim. Acta 311 (1995) 383.
[12] K.J. Williams, S.A. Thorpe, S.L. Reynolds, Int. J. Environ. Anal. Chem. 65 (1996) 149.
[13] D. Barcelo, M.-C. Hennion (Eds.), Trace Determination of Pesticides and Their Degradation Products in Water, Elsevier, Amsterdam, 1997.
[14] M. Franek, V. Kolar, S.A. Eremin, Anal. Chim. Acta 311 (1995) 349.
[15] R.M. Fuoss, U.P. Strauss, J. Polym. Sci. 3 (1948) 246.
[16] B.B. Dzantiev, A.N. Blintsov, A.F. Bobkova, V.A. Izumrudov, A.B. Zezin, Doklady Biochem. 342 (1995) 77.
[17] A.V. Zezin, V.A. Izumrudov, V.A. Kabanov, Makromol. Chem. 26 (1989) 249.
[18] B.B. Dzantiev, A.V. Zherdev, O.G. Romanenko, L.A. Sapegova, Int. J. Environ. Anal. Chem. 65 (1996) 95.
[19] M.H. Goodrow, R.O. Harrison, B.D. Hammock, J. Agric. Food Chem. 38 (1990) 990.
[20] C. Wittmann, B. Hock, Food Agric. Immunol. 1 (1989) 211.
[21] P.K. Nakane, A. Kawaoi, J. Histochem. Cytochem. 22 (1974) 1084.
[22] C. Mouvet, S. Broussard, R. Jeannot, C. Maciag, R. Abuknesha, G. Ismail, Anal. Chim. Acta 311 (1995) 331.