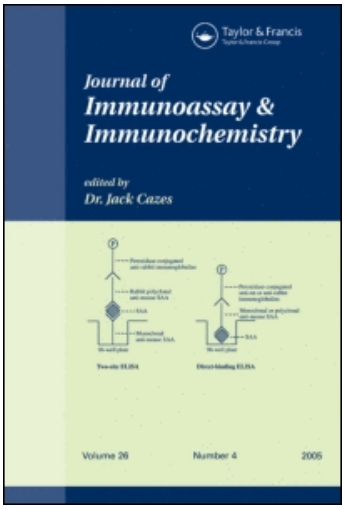

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Vadim V. Shmanai[a]
[a] Institute of Physical Organic Chemistry, Belarussian Academy of Sciences, Minsk, Belarus

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BLOCKING OF NON-SPECIFIC SORPTION IN ELISA ON FORMYLATED POLYSTYRENE BEADS

Vadim V. Shmanai
Institute of Physical Organic Chemistry, Belarussian Academy of Sciences, Surganov str.13, Minsk 220072, BELARUS

ABSTRACT

The effect of pH of the blocking solution on the non-specific sorption of peroxidase conjugates to the 0.63-cm formylated polystyrene beads was studied. Anti-G-HRP and BSA-HRP sorption was shown to dramatically depend on pH of blocking protein solutions and was minimum at pH=pI. Sorption of G-HRP weakly depended on pH of the blocking buffer. The blocking efficiency of proteins on unmodified beads, as well as of Tween-20 on formylated beads appeared to be pH independent. The optimized blocking step resulted in a multiple decrease in the non-specific sorption of conjugates on formylated beads.

(KEY WORDS: ELISA, solid phase, polystyrene beads, covalent immobilization, non-specific sorption, peroxidase conjugates)

The correspondence should be addressed to the Laboratory of Ion Exchange and Sorption, IPOCh BAS, Surganov str., 13, Minsk 220072, Belarus. E-mail: ifoch@ifoch.bas-net.by

INTRODUCTION

A method of the human immunoglobulin G solid phase radioimmunoassay (1) was based on the hydrophobic sorption of antibodies on the surface of polystyrene tubes in carbonate buffer at pH 9-10. During the following years immobilization of antigens and antibodies on various polymeric materials have been studied. These research created the basis for optimizing immunosorbents production (2).

For example, the treatment of antibodies with an acidic buffer increased their hydrophobicity (3). The following immobilization was carried out under mild conditions in order to reduce the antibodies denaturation. This increased the sensitivity of the analysis (3, 4). The authors used phosphate buffer, pH 7.4, but better results were obtained with 0.1 M acetate buffer, pH 5.0, although the choice of this particular buffer was not discussed. Some other attempts to modify classical buffers by varying their composition and pH to enhance the coating and blocking efficiency appeared to be successful (see, e.g. (5)).

The immobilization of neutral proteins for blocking of non-specific sorption has been less studied, but the problem is no less

important (6). As a rule, the same carbonate buffer or phosphate buffered saline, pH 7.2-7.4 (PBS) were used, the nature of the blocking agent being varied. Usual blocking agents are non-specific sera, non-ionic detergents, milk proteins, products of proteins partial hydrolysis, and some individual proteins such as BSA, ovalbumine, gelatin, as well as mixtures of these components.

The purpose of the present work was to study the non-specific sorption of peroxidase conjugates (NSC) in enzyme immunoassay with respect to pH of the blocking solution using the 0.63-cm formylated polystyrene beads (FPSB) as a solid phase.

MATERIALS AND METHODS

Reagents

Bovine serum albumin, fraction V (BSA) from *J.T.Baker* (The Netherlands); human immunoglobulin G (IgG) from *Belarussian blood transfusion station* (Belarus); trypsin from *Spofa* and dimethylsulfoxide (DMSO) from *Lachema* (Chech Republic); horseradish peroxidase, RZ 2.7 (HRP), and ovalbumine from *Biolar* (Latvia); sheep antiserum to IgG (Anti-G) from *Gamaleya Institute*, Academy of Sciences (Russia); Tween-20 from *Bio-Rad* (USA); and 3,3',5,5'-tetramethylbenzidine (TMB) from *Serva* (Germany) were

used. Buffer solutions were prepared from salts, acids, and alkalis from *Reachim* (Russia).

Rabbit antiserum to IgG was obtained by the conventional method (7) with IgG purified by gel-filtration on a Sephacryl S-300 (*Pharmacia*, Sweden) column (2×100 cm) in PBS; antibodies were ammonium sulfate reprecipitated four times; DMSO was distilled at low pressure; horse serum was heated for 0.5 h at 56°C, centrifuged, and filtered through a 0.2-μm membrane (*MIFIL*, Belarus). Other materials were used without additional purification.

Initial solutions of BSA in water, IgG and Anti-G in PBS, and trypsin in 0.1 M phosphate buffer, pH 10, contained the protein in concentration 100 mg/mL and 0.1% sodium azide. Blocking solutions were prepared by dilution of the initial solutions with the required buffer; if necessary, pH value was brought to the desired level with 1 M HCl or NaOH.

Conjugates Anti-G-HRP, Anti-G-(rabbit)-HRP, BSA-HRP, and IgG-HRP were prepared by the periodate method (8). Substrate solution for measuring the HRP activity contained 1 mM TMB and 2.5 mM hydrogen peroxide in 15% DMSO and was prepared by mixing the TMB and H_2O_2 solutions directly before the analysis.

Beads

The surface layer of the injection molded polystyrene balls was modified with aldehyde groups by the method previously used in the formylation of macroreticular polystyrene (9). Earlier we described the FPSB as a matrix for the immobilization of substances containing primary amino groups, including proteins (10, 11). The content of the active groups (1.01±0.02 micromol/cm^2) was determined with 2,4-dinitrophenylhydrazine by a specially developed method (12).

Sorbent Processing with the Blocking Agent

Beads were placed in the blocking solution (1% protein or 0.1% Tween-20; 0.2 mL/bead); the mixture was gently shaken for 4 h at room temperature and then for 16 h at 4°C. The beads were washed three times with PBS for 0.5 h and stored in PBS before using.

Determination of NSC

The beads previously processed with a blocking agent were placed into polystyrene test-tubes 10×75 mm; 0.200 mL of the peroxidase conjugate in PBS containing 0.05% Tween-20 (PBS-T) was added to all test-tubes, and the incubation was performed at constant shaking at 25°C on a water bath for 30 min. The liquid was

aspirated, and beads were washed with 1 mL PBS-T, 5 mL water, and two times with 1 mL water (total washing time 10 min). Substrate solution (0.250 mL) was added, and the test-tubes were shaken for 15 min at 25°C. The enzymic reaction was stopped by adding 1 mL 0.5 M sulfuric acid; the optical densities (ODs) were measured at 450 nm and 1 cm layer in a photocolorimeter "Spekol 221" *Carl Zeiss JENA* (Germany). When the OD value exceeded 2.0, the solution was diluted 10 times with 0.4 M sulfuric acid and measured again. All experiments were carried out in triplicate and the average results reproducibility was ±10%.

RESULTS

The values of the Anti-G-HRP non-specific sorption (initial concentration 500 ng/mL) on the FPSBs blocked by BSA, ovalbumine, horse serum, or Tween-20 in 0.1 M phosphate buffer, pH 4 to 10, are shown in Figure 1. NSC was expressed as the OD of the substrate solution after the enzymic reaction of the sorbed conjugate. NSC values on beads blocked by BSA and ovalbumine were minimum at pH about 5, and grew substantially with the increase in pH of the blocking solution. Beads blocked by horse serum showed the minimum NSC level at pH 5, but no increase of NSC in alkaline

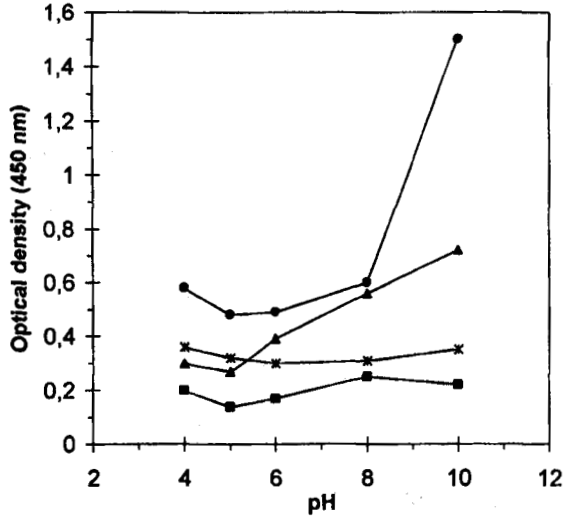

FIGURE 1. Effect of pH of the blocking solution on non-specific sorption of Anti-G-HRP (500 ng/mL). FPSB were blocked with horse serum (squares), Tween-20 (stars), ovalbumine (triangles) and BSA (circles) in 0.1 M phosphate buffer.

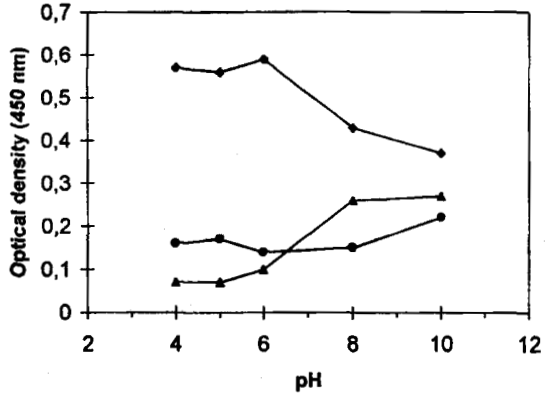

FIGURE 2. Effect of pH of the blocking solution on non-specific sorption of G-HRP (1000 ng/mL). FPSB were blocked with BSA (circles), ovalbumine (triangles) and trypsin (rhombs) in 0.1 M phosphate buffer.

conditions was observed. pH value of the Tween-20 blocking solution did not influence the NSC level.

The results of similar experiments with G-HRP (initial concentration 1000 ng/mL) are shown in Figure 2. The use of horse serum, Tween-20 (data are not shown), and BSA gave the unchanged NSC level at different pH, but ovalbumine in weakly alkaline buffers resulted in a threefold NSC increase. On the contrary, blocking with trypsin appeared to be the most efficient at pH 10.

The most significant influence of pH of the blocking solution on the NSC level was observed with BSA-HRP (500 ng/mL, Figure 3). Both individual proteins (BSA, ovalbumine) and horse serum showed the minimum NSC at pH 4-5 and its dramatic increase with the enhancement of pH from 5 to 10: sixfold increase in the case of BSA, fourfold increase for ovalbumine, and threefold increase for the horse serum blocked beads. NSC on the FPSB blocked by Tween-20, like in the previous experiments, was not pH dependent in all investigated ranges.

To compare the FPSB and unfunctionalized polystyrene beads (SPSB) intended for the passive adsorption of hydrophobic proteins, we studied NSC of three above mentioned conjugates on both bead

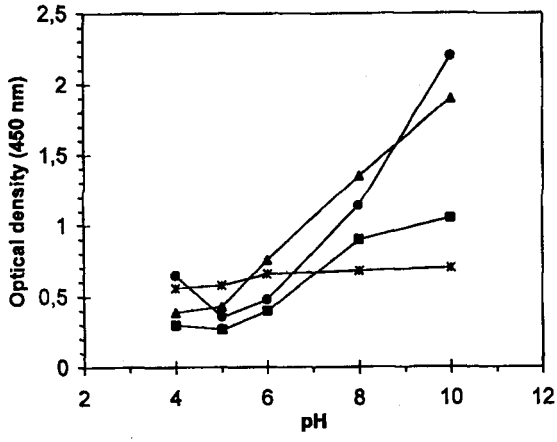

FIGURE 3. Effect of pH of the blocking solution on non-specific sorption of BSA-HRP (500 ng/mL). FPSB were blocked with Tween-20 (stars), horse serum (squares), ovalbumine (triangles) and BSA (circles) in 0.1 M phosphate buffer.

types. Its values on SPSB blocked by the various agents or without any preliminary blocking were in the range of 0.05-0.1 without expressed pH dependence.

Taking into account the fact that the FPSB contains functional groups reacting with proteins, we assumed that some decrease of NSC could be caused by adding of neutral proteins into the conjugate solution. Results of the BSA-HRP (500 ng/mL) sorption in the presence of BSA or horse serum are given in Table 1.

NSC was studied with respect to the initial conjugate concentration. The most effective blocking step for the FPSB was

TABLE 1

NON-SPECIFIC SORPTION of BSA-HRP (500 ng/mL) on POLYSTYRENE BEADS

Conjugate Solvent	Beads			
	SPSB	FPSB	FPSB-HS**	FPSB-BSA**
PBS-T	0.07	12.50	0.26	0.40
PBS-T-BSA*	0.06	6.22	0.08	0.12
PBS-T-HS*	0.04	6.38	0.13	0.10

* Protein concentration 1%.

** The beads were treated with 1% protein solution in 0.1 M phosphate buffer, pH 5.0.

TABLE 2

DIRECT IMMUNOASSAYS of IgG-HRP and Anti-IgG-HRP

Beads	Conjugate concentration, ng/ml	OD_{450}	Background/ signal,%
FPSB-IgG	20	0.808±0.024	2.2
	100	3.140±0.040	3.2
FPSB-IgG	20*	0.618±0.021	0.6
	100*	2.340±0.091	0.8
FPSB-Anti-IgG	100	0.820±0.035	1.2
	1000	6.490±0.220	1.8

*Anti-IgG-(rabbit)-HRP was used.

used: the beads were treated with 15% horse serum in 0.1 M phosphate buffer, pH 5.0, the conjugates were dissolved in PBS-T-BSA. Linear sorption isotherms of three conjugates were obtained at up to 2 γ/mL initial concentration.

Direct immunoassays of IgG-HRP on Anti-IgG-coated FPSB and of Anti-IgG-HRP on IgG-coated beads were carried out (Table 2). The working concentrations of the conjugates (20-200 ng/mL) gave NSC values in the range of 0.005-0.02.

DISCUSSION

Covalent attachment of antibodies or antigens to solid supports was known to be more effective than hydrophobic sorption because of higher binding stability (13). Immobilized proteins denaturated less and were not lost during the assay. As a consequence, chemically activated supports allowed the sensitivity and reproducibility of the analysis to be increased (6). The FPSBs were applied as a matrix for immunochemical analysis of steroid hormones and strophanthin K (14), and enzyme immunoassay with improved thermostability of immobilized antibodies (15). In "sandwich"-type ELISA of hCG, the FPSBs showed more reproducible results than SPSBs (2% versus 6%) with the zero point as low as 0.005 after the optimized blocking step (author's data).

The FPSB did not require activation prior to protein immobilization nor condensing agents in the course of the reaction, and could be stored at least for five years without deactivation.

Moreover, a wide range of possibilities have been opened, such as covalent grafting of spacer arms of a desired length and structure; site-specific binding of antibodies for immunoassay by an appropriate method; easy immobilization of peptides and amino acids (10).

However, non-specific sorption of peroxidase conjugates on FPSB-based immunosorbents appeared to exceed that on SPSB. Was it possible to reach NSC on the FPSB as low as on SPSB to use all the advantages of formylated beads and thereby avoid their drawbacks?

Proteins chemisorption on the FPSB was efficient at different pH in various buffers not containing primary amines (16). The maximum density of immobilized protein (DIP) was reached at the pH corresponding to the isoelectric point of protein (pI). DIP of IgG on the FPSB was 1.5 times greater at pH 6.0 than at 9.5, whereas DIP of albumins turned out to be the most pH dependent. DIP of BSA was 3-4 times higher at pH 4.6 than at pH 3.0 or 9.0, and twice as high as at 7.4. Therefore, it was interesting to investigate the efficiency of the blocking step as a function of pH with respect to the non-specific peroxidase conjugate sorption. It would help to find the best blocking conditions. We blocked beads with no preliminary immobilized antibodies to avoid their possible interaction with conjugates. To

obtain reliable ODs, conjugates were taken in concentration 500-1000 ng/mL, which was several times greater than concentrations usually applied in analysis.

It is hardly surprising that the most effective blocking occurred at the solution pH corresponding to the protein's pI. On the contrary, the lack of the pH influence on the blocking efficiency seems to be surprising. Proteins have a reduced size of the molecules and do not have electrostatic repulsion at pH=pI. As a result, they gave maximum DIP under these conditions. The following pH change to 7.4 should magnify the size of molecules. Two processes seem to be possible: an increase in the protein layer thickness as the protein's conformation allows, or desorption (expelling) of weakly bound molecules. The first mentioned process may result in a decrease in the NSC due to the swelling of the protein layer. This leads to a poorer availability of the binding sites on the support surface for the conjugate molecules. A similar process can not be expected for the sorbed non-ionic Tween-20. Desorption is known for physically sorbed proteins, but it is not likely to happen to the covalently immobilized proteins.

Actually, formylated beads treated with BSA or ovalbumine showed minimum of the BSA-HRP sorption at pH of the blocking

solution in a range of 4-5, which corresponded to pI of these proteins. Horse serum gave the same minimum, because albumins are the major components of sera. The sorption of Anti-G-HRP on FPSB-BSA remained unchanged at pH of the blocking solution from 4 to 8, but increased at pH 10. And, finally, ovalbumine and trypsin but not BSA and horse serum showed some pH dependence of IgG-HRP sorption on the blocked beads.

Inactivated trypsin has not been used before as a blocking agent. However, it was interesting to study the pH influence on this protein's capacity for NSC blocking, because of its pI equal to 10.8. The G-HRP sorption on the FPSBs blocked with trypsin was found to be minimum at pH 10. It confirmed the assumption that the best blocking effect was observed at the pH corresponding to pI of the protein.

As has been reported earlier (17), the addition of a non-ionic detergent to the conjugate solution allowed the blocking stage to be excluded for unmodified polystyrene. We obtained similar results: non-blocked SPSB as well as the beads blocked at various pH showed similar NSC (less than 0.01) in a PBS-T-based conjugate solution. As to the FPSB, a preliminary blocking step appeared to be necessary.

Non-specific proteins added to the conjugate solution caused a further NSC lowering to 0.05-0.15. These values corresponded to NSC on SPSBs. Direct immunoassay of the conjugates studied showed a good background-to-signal ratio (1.5% on the average) and an NSC level for conjugated rabbit antibodies by several times lower than for sheep antibodies (Table 2).

The reduction of the initial concentration of a conjugate causes a substantial NSC decrease, but worsens the specific reaction. Compromises have been found between the highest allowed conjugate concentration and lowest possible NSC level for the best assay sensitivity. The linear sorption isotherms made it possible to easily select the suitable concentrations of the conjugate.

In conclusion, the level of non-specific sorption of peroxidase conjugates is controlled by the properties of all components of the assay: the solid support, the blocking solution, the conjugate, and the buffer. Therefore, optimum conditions of the blocking step should be selected in the course of special experiments for each specific case. The general approach to the problem related to the FPSB was formulated as follows: (1) The blocking step with the most suitable proteins is the least effective in conventional alkaline buffers,

therefore, the carbonate buffer should not be used. (2) The blocking efficiency is improved if buffers with the pH equal to the pI of the blocking protein are used. (3) The presence of neutral proteins in the conjugate solutions (in addition to detergents) suppresses the non-specific sorption on the FPSB.

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