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Oriented antibody immobilization to polystyrene macrocarriers for immunoassay modified with hydrazide derivatives of poly(meth)acrylic acid

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Abstract

Background: Hydrophobic polystyrene is the most common material for solid phase immunoassay. Proteins are immobilized on polystyrene by passive adsorption, which often causes considerable denaturation. Biological macromolecules were found to better retain their functional activity when immobilized on hydrophilic materials. Polyacrylamide is a common material for solid-phase carriers of biological macromolecules, including immunoreagents used in affinity chromatography. New macroformats for immunoassay modified with activated polyacrylamide derivatives seem to be promising.

Results: New polymeric matrices for immunoassay in the form of 0.63-cm balls which contain hydrazide functional groups on hydrophilic polymer spacer arms at their surface shell are synthesized by modification of aldehyde-containing polystyrene balls with hydrazide derivatives of poly(meth)acrylic acid. The beads contain up to 0.31 μmol/cm^2 active hydrazide groups accessible for covalent reaction with periodate-oxidized antibodies. The matrices obtained allow carrying out the oriented antibody immobilization, which increases the functional activity of immunosorbents.

Conclusions: An efficient site-directed antibody immobilization on a macrosupport is realized. The polymer hydrophilic spacer arms are the most convenient and effective tools for oriented antibody coupling with molded materials. The suggested scheme can be used for the modification of any other solid supports containing electrophilic groups reacting with hydrazides.

Background

Polyacrylamide is a common material for solid-phase carriers of biological macromolecules, including immunoreagents used in affinity chromatography and immunoassay [1,2]. Microparticles obtained by the acrylamide and methylene-bis-acrylamide copolymerization require amide group activation [3]. Various schemes of polyacrylamide modification to introduce applicable functional groups for covalent protein immobilization were reported: hydrolysis to form carboxylic groups (the immobilization is carried out through the protein's amino groups using condensing agents), hydrazinolysis to form hy-

drazide groups (immobilization through the aldehyde groups of the glycoprotein's carbohydrate fragments oxidized with periodate), aminolysis with bifunctional amines (condensation with the protein's carboxylic groups), modification with glutaric aldehyde (immobilization through the amino and sulfhydryl groups of a protein) [3]. Proteins are known to satisfactorily retain their stability on such matrices.

As for the solid phase ***macro*** carriers (plates, balls, tubes) in immunoassay, hydrophobic polystyrene is used as the most common material. Proteins are immobilized on polystyrene by passive adsorption, which often causes considerable denaturation [4].

Biological macromolecules were found to retain better their functional activity when immobilized through extended hydrophilic spacer arms [5]. As the sorption on this kind of matrices is substantially reduced [6], it is mandatory to use the chemical coupling of reagents. One of the causes of the evident inconsistencies of the aforementioned data is the operational problems of handling microparticles (the dosage accuracy and the carrier separation from the liquid phase) and the unavailability of moulded polyacrylamide macrocarriers.

Therefore, obtaining new macroformats modified with activated polyacrylamide spacer arms seems to be promising. This would allow one to combine the operational advantages of moulded polystyrene macrosupports with the functional advantages of polyacrylamide, so as to apply the advantages in the field of the protein immobilization achieved in enzymology and affinity chromatography [2] to immunoassay techniques.

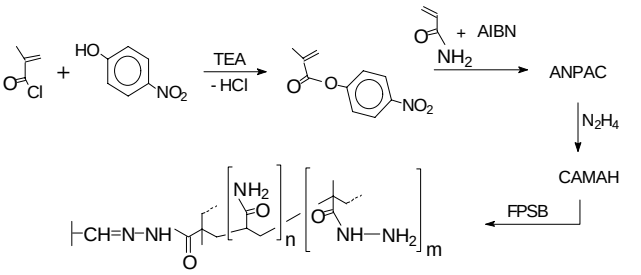

Figure 1
The scheme of chemical modification of formylated polystyrene ball (FPSB) by acrylamide/methacryloyl hydrazide copolymers (CAMAH), which were obtained by processing copolymers of acrylamide and 4-nitrophenyl methacrylate (ANPAC) with hydrazine. A molar ratio m:n = 1:9 (CAMAH-1); 1:2 (CAMAH-2); 1:1 (CAMAH-3).

The aims of the present paper are: syntheses of activated poly(meth)acrylic acid derivatives to modify the surface of previously described 0.63-cm diameter formylated polystyrene balls (FPSB) [7], as well as oriented antibody immobilization on modified balls in order to obtain advanced immunosorbents.

Results and discussion

The syntheses of carriers modified with polymeric hydrazides are based on reactions presented in Figure. The results of the experiments are depicted in Table 1. The presence of 33% hydrazide compound in the initial monomer mixture appeared to be sufficient to achieve the maximum content of active groups. Copolymer of such composition showed a greater hydrazide group activity in solution, as well as a greater binding ability in comparison with copolymers containing 10 or 50% hydrazide groups.

Table 1: Preparation and properties of polystyrene ball modified with copolymers of acrylamide and methacryloyl hydrazide

Copolymer	4-Nitrophenyl acrylate content, mol.%	Nitrogen content in the copolymer by elemental analysis, % (theoretical value)	Copolymer activity by the reaction with TNBS*, OD_{520}	Content of active hydrazide groups on a carrier (μmol/cm^2), determined	
				by the decrease in the copolymer content in solution	by the reaction of ball with TNBS
CAMAH-1	10	19.1 (20.8)	0.39	0.42	0.06
CAMAH-2	33	24.6 (23.1)	1.42	1.23	0.31
CAMAH-3	50	24.5 (24.5)	1.28	1.05	0.31

* 0.2 ml of a copolymer solution (0.1 mg/ml) was added to 1 ml of $2.5 \cdot 10^{-3}$ M TNBS solution in 0.1 M borax. The solution obtained was allowed to stay for 15 min, and then OD_{520} was measured.

Oriented antibody immobilization

Antibody molecules (immunoglobulins G) are known to have Y-shape and to consist of one Fc and two Fab fragments [8]. Fab fragments react with antigens to yield immune complexes, so their spatial accessibility is an important factor in ensuring the immunosorbent activity. Depending on the immobilized molecule orientation on the carrier surface, a higher or lower amount of Fab fragments appears to be available for the interaction with the antigen present in the solution. The mode of antibody immobilization determines its orientation, and, as a result, its activity in immunoassay can vary. Random antibody immobilization occurs in the case of hydrophobic sorption, as well as when carriers containing the aldehyde, iminoester, halogeneacetyl, or other electrophilic groups are used to react with protein amino groups. Furthermore, results were published that demonstrate that amino groups of the antigen-binding site show higher chemical activity over other ones within the immunoglobulin molecule [9]. Several techniques of the directed antibody immobilization on microparticles were developed to improve the immunosorbent activity [10]. They take into account the features of antibody molecular structure, as well as the differences between Fab and Fc fragments. The most known scheme includes oxidation of carbohydrate residues in the Fc fragment of immunoglobulin followed by the reaction of the resulting aldehyde groups with hydrazine or hydrazide groups of the carrier [11].

An attempt to immobilize oxidized antibodies on amino-containing carriers was made [12]. This approach is not assumed to be satisfactory, since the reaction is to be carried out in alkaline media. The aldehyde groups of the protein easily react with more available amino groups of other protein molecules in solution. This causes formation of large, covalent protein complexes. As a result, the accessibility of Fab-fragments appears to be low even in the case of a high density of the protein immobilization. At the same time, hydrazide groups react with aldehydes in a low acidic medium, where the equilibrium of the aldehyde-and-amine reaction is significantly shifted to the initial compounds. Thus, the influence of this side reaction is decreased. Radiation refinement of the molded polystyrene surface with a low-molecular hydrazide was realized, but the results do not seem to be convincing [13].

We investigated two types of carriers, which were produced by a modification of aldehyde groups on the surface of formylated polystyrene balls: (1) with a range of low-molecular-weight hydrazide spacer arms described in our previous paper [14], and (2) with copolymers of acrylamide and methacrylic acid hydrazide. The results are given in Table 2.

Table 2: Oriented anti-IgG immobilization on hydrazide containing carriers

Carrier	Content of active hydrazide groups, $\mu mol/cm^2$	Immunosorbent activity, OD_{450}	
		with oxidized antibody	with non-oxidized antibody
FPSB	0	0.71	0.61
FPSB-PTH	0.64	0.63	0.37
FPSB-CAMAH-1	0.09	0.51	0.18
FPSB-CAMAH-2	0.14	0.84	0.30
FPSB-CAMAH-3	0.13	0.79	0.28
FPSB-PMAH	0.11	0.53	0.16

An increase in the immunosorbent activity was observed in all the experiments when oxidized antibodies were immobilized on the hydrazide-containing carriers, as compared with control experiments with non-oxidized antibodies. However, low-molecular-weight hydrazides appear to be poorly suited for these purposes, as a high activity of carriers treated with non-oxidized antibodies was observed. We believe that the reason thereof is non-specific hydrophobic sorption of antibodies. PTH-modified FPSB appeared to be the best among malonyl-, glutaryl, adipoyl, azeloyl, D-tartaroyl, and tetraoxahexadecane-1,16-dioyl hydrazides, but even its non-specific sorption amounted to 60%. Such carriers could be used for the immobilization of hydrophilic antigens, which display a low physical adsorbability. On the contrary, synthesized copolymers ensure a high degree of hydrophilization of the modified surface, and long-chain reactive groups can easily react with aldehydes in antibody molecules with minimum steric hindrances.

Thus, we observed a pronounced effect of directed immobilization on carriers modified with polymeric spacer arms. Our results support the data of Hoffman and O'Shannessy [15], who synthesized microparticles for affinity chromatography that contained hydrazide groups on spacer arms 10, 15, and 21 atoms long. An increase in the length of the spacer arm enhanced the activity of directedly immobilized antibodies. Thus, polymeric spacer arms seem to be the logical continuation of this series of supports to obtain an optimal matrix.

The best result in our work was shown by the sorbent modified with hydrophilic copolymer CAMAH-2. Activity of immobilized antibodies was 38 percent higher than that of randomly covalently immobilized antibodies to

FPSB. And the latter were still more active than antibodies adsorbed on unmodified beads by hydrophobic interaction (results are not shown).

The hydrazide carriers presented in Table 2 were modified with glutaraldehyde to perform the purely random covalent immobilization via amino groups of the antibody. The activity of immunosorbents diminished by 2–3 times.

Conclusions

New polymeric matrices for immunoassay in the form of 0.63-cm balls containing hydrazide functional groups on hydrophilic polymer spacer arms at their surface shell were synthesized. The obtained matrices allow carrying out the oriented antibody immobilization, which increases the functional activity of immunosorbents. An analysis of the results and their comparison with the published data allow one to state that the polymer hydrophilic spacer arms are the most convenient and effective tools for oriented antibody immobilization on molded materials. The suggested scheme can be used for the modification of any other solid supports that contain electrophilic groups reacting with hydrazides.

Materials and methods

Hydrazine hydrate, 4-nitrophenol, triethylamine, and methacryloyl chloride were obtained from Reachim (Russia). 2,4,6-Trinitrobenzenesulphonic acid (TNBS) and horseradish peroxidase HRP were purchased from Biolar (Latvia). Acrylamide, Acrylex (analog of Bio-Gel) P6, and sodium metaperiodate were obtained from Reanal (Hungary). 3,3',5,5'-Tetramethylbenzidine (TMB) was obtained from Serva (Germany). Distilled triethylamine and freshly distilled methacryloyl chloride were used. Hydrazine hydrate was titrated with Methyl Orange. We have previously reported on the purification of human immunoglobulin G, as well as production and properties of its HRP conjugate (IgG-HRP), rabbit antibodies to IgG (Anti-IgG), and 1,1,3,3-propanetetracarbohydrazide (PTH) [7,14,16]. The HRP-catalyzed oxidation of TMB with hydrogen peroxide was used as an indicator reaction. This was quenched with 0.5 M sulfuric acid, and the immunosorbent activity was evaluated by the optical density of the resulting solution at 450 nm.

We synthesized copolymers of acrylamide and the activated ester of methacrylic acid. The nitrophenol residue of this ester can be replaced with a variety of functional groups in mild conditions. p-Nitrophenyl methacrylate was produced by the reaction of methacryloyl chloride and p-nitrophenol in the presence of triethylamine according to [17]. Copolymerization of 4-nitrophenyl methacrylate and acrylamide was conducted in a mixture of equal volumes of anhydrous acetone and benzene. Azodiisobutyronitrile (0.3 g) was added to a solution of 2.44 g (0.012 mol) of 4-nitrophenyl methacrylate and 7.56 g (0.106 mol) of acrylamide. The solution was refluxed for 22 h with stirring. The precipitated copolymer was then filtered off, washed twice with 50 ml of methanol, and dried under vacuum to give 8.4 g of the product (ANPAC-1). Copolymers with the acrylamide to 4-nitrophenyl methacrylate molar ratios 2:1 (ANPAC-2) and 1:1 (ANPAC-3) were similarly obtained.

To produce the acrylamide/methacryloyl hydrazide copolymers, 1 g of ANPAC was dissolved in 5 ml of hydrazine hydrate, stirred for 2 h at room temperature and then for 3 h at 40–45°C, and left overnight. Hydrazine was distilled off under vacuum. The residue was twice evaporated under vacuum with 10-ml portions of water to completely remove hydrazine. Then 5 ml of water was added, the resulting solution was acidified with acetic acid up to pH 5, and 50 ml of ethanol was added dropwise. The precipitate was washed with ethanol and reprecipitated. The product (copolymer of acrylamide and methacryloyl hydrazide, CAMAH) was separated by centrifugation and investigated by elemental analysis and the reaction with TNBS [14].

Commercial polymethylmethacrylate (acrylic plastic) was precipitated from a benzene solution with methanol, washed, and boiled in a large excess of hydrazine hydrate until complete dissolution. Hydrazine was removed, and polymethacryloyl hydrazide (PMAH) was twice precipitated with ethanol from an aqueous solution.

Reaction of CAMAH (5 mg/ml) with aldehyde-containing balls was conducted for 4 h in 0.1 M phosphate buffer, pH 5.0 (0.2 ml/ball) and shaking. The balls were then completely washed from the hydrazide.

We performed the quantitative analysis of the hydrazide groups located on the surface of the carriers obtained. TNBS is known to react with hydrazides, and the decrease in its content was used for the analysis. The ball to be examined was shaken for 1.5 h in 300 µl of a freshly prepared TNBS solution in a 0.1 M sodium tetraborate solution, pH 9.3, at room temperature. The solution was removed; the ball was placed into 300 µl of the borax solution and allowed to stay for 5 min with continual shaking to completely wash out the adsorbed reagent. The solutions were pooled, and a 200-µl aliquot of the mixture was mixed with 1 ml of 5 mM hydrazine solution in 0.1 M borax. The mixture was stirred and allowed to stay for 15 min at room temperature. The same was performed with unmodified FPSB and the initial TNBS working solution without a ball. OD values were measured at 520 nm in a 1-cm cell. The TNBS solutions were found to satisfy the Beer law in the range of investigated

concentrations. Therefore, no calibration was needed, and the following formula was used for the calculations:

$$\mathbf{A\ (mol/cm^2) = (OD_2 - OD_3) \cdot c \cdot V / OD_1 \cdot S}$$

$\mathbf{OD_1}$ – optical density of the initial solution without a ball;

$\mathbf{OD_2}$ and $\mathbf{OD_3}$ – optical densities of solutions from the experiments with the initial ball (FPSB) and hydrazide-containing one, respectively;

c – concentration of the TNBS working solution, mol/l;

V – volume of the TNBS working solution, l;

S – ball surface area, cm^2.

Antibodies were oxidized with 0.1 M $NaIO_4$ solution in 0.1 M acetate buffer (pH 5). The protein was isolated by gel-filtration on an Acrylex P6 column (2 × 10 cm) in the same buffer solution. The oxidized antibodies were immobilized with continual shaking at room temperature for 4 h and then overnight at 4°C. The balls were washed, and the activity was determined by means of the direct enzyme immunoassay of IgG-HRP conjugate.

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