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ORIGINAL CONTRIBUTION

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Attachment of horseradish peroxidase (HRP) onto the poly(styrene/acrolein) latexes and onto their derivatives with amino groups on the surface; activity of immobilized enzyme

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Abstract The polystyrene (P(S)), poly(styrene/acrolein) (P(SA)), and polyacrolein (P(A)) latexes, with varied fraction of polyacrolein in the surface layer (f_A = 0, 0.50, 0.63, 0.84, 1.00), were used for the attachment of horseradish peroxidase. Surfaces of latexes were modified by reaction with ethylenediamine. In this step the aldehyde groups from polyacrolein were blocked and the primary amino groups were introduced. The carbohydrate portion of HRP was oxidized in the reaction leading to formation of aldehyde groups. The adsorption and covalent immobilization of HRP onto the P(S), P(SA), and P(A) latexes and of the oxidized HRP (HRP-OX) onto the modified latex particles, with amino groups on the surface (P(SA)-M and P(A)-M), were investigated. The activities of parent and oxidized HRP were compared with activities of the corresponding enzymes in solution. It has been found that whereas HRP is not suitable for the covalent immobilization on P(SA) latex and loses its activity after adsorption onto P(S) latex, HRP-OX can be adsorbed onto P(S) latex and is readily immobilized covalently onto the ethylenediamine modified P(SA) and P(A) latexes, retaining much of its former enzymatic reactivity.

Key words poly(styrene/acrolein) latex – horseradish peroxidase – activity of immobilized enzyme

Introduction

Latexes, polymeric beads, and microspheres bearing attached proteins on their surfaces are often used as important tools in basic studies in life sciences [1–4], medical diagnostics [5], and as supports in biotechnology [6]. The ideal polymeric supports should allow to attach proteins in a controlled manner, resulting in the required surface concentration of the covalently immobilized (or, in some cases, adsorbed) proteins retaining a maximum of their biologic activity. Many kinds of latexes, with various reactive groups on the surface suitable for the covalent immobilization of proteins, have been synthesized. Some reactive groups require activation prior to the protein immobilization (e.g. –OH groups should be activated with cyanogen bromide and –COOH groups with carbodiimides [7, 8]). Some other (e.g., aldehyde [9–13] and succinimide [14, 15]) allow direct protein immobilization, without activation. In many instances it was found that adsorption and/or covalent immobilization of proteins, particularly enzymes, onto the solid supports leads to considerable reduction or even to complete loss of their activity [16–20]. For a given protein the maximal attainable surface concentration of immobilized protein and the retention of protein biologic activity upon immobilization strongly depend on the nature of polymeric particles and on the procedures used for immobilization. In this paper

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we describe studies of the adsorption and covalent immobilization of horseradish peroxidase (HRP) (in its commercially available and oxidized forms) onto the poly(styrene/acrolein) latexes and onto their derivatives with the amino groups on the surface. For each kind of enzyme-latex system we also compare activities of the attached enzymes and enzymes in solution. HRP was chosen for our studies because of its applications in diagnostic assays [21–23].

The polyacrolein-containing latexes, first synthesized for biological and medical applications by Rembaum [1, 10, 13] and Margel [9, 11, 24], were found to be suitable for direct immobilization of protein macromolecules via Schiff's base linkages formed in reaction between the aldehyde groups of polyacrolein and amino groups of proteins. Rembaum et al. found that the acrolein-2-hydroxyethyl methacrylate copolymer microspheres could be used for the covalent immobilization of HRP in the active form and observed the dependence of the activity of immobilized HRP-microsphere system on the bulk composition of the polymeric particles [19]. Unfortunately, data presented in the mentioned papers do not allow to evaluate the role of variations of the surface concentrations of immobilized HRP and of its specific enzymatic activity in the overall activity of the protein-microsphere system. Moreover, the activity of HRP on the poly(acrolein/2-hydroxyethyl methacrylate) microspheres was related to the bulk composition of particles and not to the composition of the particle surface being in contact with HRP.

Recently, we synthesized several poly(styrene/acrolein) latexes (P(SA)) with the well characterized composition of the surface layer [25]. These P(SA) latexes were found to be suitable for the attachment of human serum albumin and gamma globulins with the controlled balance, depending on the nature of the surface layer of latex particles, of the adsorption and covalent immobilization of protein macromolecules [26, 27]. By using the P(SA) latexes and their derivatives for which the aldehyde groups on the surface were replaced with the amino groups, we expected to establish the relations between the surface concentrations of adsorbed and immobilized HRP, activity of the attached enzyme, and the chemical composition of the surface layer of latex particles.

Experimental Part

Materials

Latexes

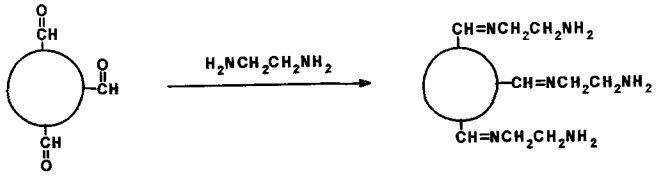
The polystyrene (P(S)), poly(styrene/acrolein) (P(SA)), and polyacrolein (P(A)) latexes were obtained in the radical homopolymerization and/or copolymerization of styrene and acrolein initiated with $K_2S_2O_8$ and carried out without emulsifier. The details of the synthetic procedure and methods used for latex characterization are given in our earlier paper [25]. The P(SA) latexes obtained according to this method have the core-shell structure with core rich in polystyrene and shell enriched in polyacrolein.

Modification of the P(SA) and P(A) latexes, resulting in blocking the aldehyde groups and introduction of the primary amino groups, was performed in reaction involving the appropriate latex particles and ethylenediamine. The overall process of modification is illustrated in Scheme I. The detailed recipe is given below. Latex particles in 15 mL of latex suspension (6% wt/vol) were transferred to ethanol by the four times repeated centrifugation (15 000 G) and resuspension in ethanol. Ethylenediamine was added in excess (100 times molaly with respect to the surface aldehyde groups) to the suspension of latex in ethanol (20 mL, latex concentration 4.8% wt/vol). The mixture was shaken for 15 h at room temperature. According to our previous findings, this excess of ethylenediamine prevents the interparticle bridging resulting in particle aggregation [28]. Particles labeled with ethylenediamine were transferred to pure ethanol (20 mL) and the Schiff's

Table 1 Parameters characterizing P(S), P(SA) and P(A) latexes (data from ref. [25]).

Latex	$\overline{D}_n$ μm	$\overline{D}_v/\overline{D}_n$ [a]	Fraction of polyacrolein in latex particles	Fraction of polyacrolein in the surface layer of latex particles [b]	Surface concentration of aldehyde groups [c] mol/m²	Surface concentration of acidic groups mol/m²
P(S)	0.52	1.004	0	0	0	$3.39 \cdot 10^{-6}$
P(SA)1	0.51	1.010	0.027	0.50	$2.20 \cdot 10^{-6}$	$2.52 \cdot 10^{-6}$
P(SA)2	0.49	1.002	0.13	0.63	$2.28 \cdot 10^{-6}$	$1.38 \cdot 10^{-6}$
P(SA)3	0.38	1.007	0.22	0.84	$2.51 \cdot 10^{-6}$	$2.14 \cdot 10^{-6}$
P(A)	0.30	1.003	1.00	1.00	$1.96 \cdot 10^{-6}$	$3.24 \cdot 10^{-6}$

[a]) Number averaged diameter $\overline{D}_n = \Sigma n_i D_i / \Sigma n_i$, volume averaged diameter $\overline{D}_v = \Sigma n_i D_i^4 / \Sigma n_i D_i^3$, polydispersity parameter $\overline{D}_v/\overline{D}_n$.
[b]) Determined by XPS spectroscopy
[c]) Determined by characteristic reaction with 2,4-dinitrophenylhydrazine.



Scheme

base linkages formed by reaction between amino groups from ethylenediamine and aldehyde groups from the polyacrolein segments on the surface were reduced by addition of 0.2 mL of $NaBH_3CN$. After 1h of reaction involving $NaBH_3CN$ the latex particles were transferred to ethanol and then to water by the six times repeated centrifugation and resuspension, first in pure ethanol and then in water.

The modified poly(styrene/acrolein) and polyacrolein latexes, with amino groups on the surface, are denoted as P(SA)-M and P(A)-M, respectively.

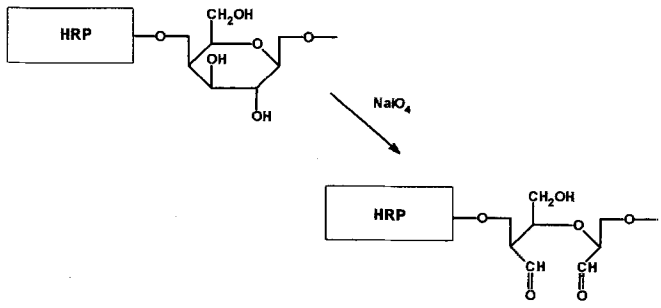
Peroxidases

Horseradish peroxidase (HRP) (Sigma, Type VI) was used as received.

Horseradish peroxidase with oxidized carbohydrate part of the enzyme (HRP-OX) was prepared in a similar manner as described by Nakane and Kawaoi [29]. Process of oxidation is outlined in Scheme II. HRP (5 mg) was dissolved in 1 mL of fresh 0.3 M sodium bicarbonate buffer (pH = 8.1). 1-Fluoro-2,4-dinitrobenzene (Sigma) (0.1 mL of 1% solution in absolute ethanol) was added and the solution was stirred during 1 h at room temperature. 1-Fluoro-2,4-dinitrobenzene was used with the purpose to block the free amino groups in HRP and thus to avoid any possible conjugation resulting from the reaction of free amino groups with aldehyde groups in the oxidized carbohydrate portion of the enzyme. In the next step 0.1 mL of 0.06 M solution of $NaIO_4$ in distilled water was added to the solution of HRP with blocked amino groups. Oxidation was carried out during 30 min at room temperature. Subsequently, the ethylene glycol (1 mL of water solution with concentration 0.16 mol/L) was added and the solution was stirred at room temperature for 1 h. The oxidized HRP was dialyzed against 0.01 M bicarbonate buffer with pH = 9.5 (1-liter portions changed every 12 h) at 4 °C. Eventually, the solutions of purified oxidized HRP were stored in Eppendorf tubes at 4 °C.

Assay Method

The activity of HRP and oxidized HRP was determined by the fixed time method based on the assay developed by

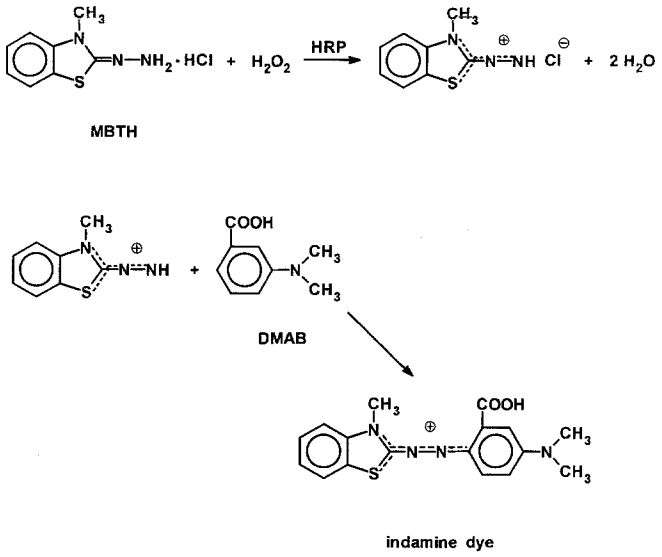


Scheme II

Ngo and Lenhoff [30]. The assay consists of two steps, first, the enzymatic oxidation of 3-methyl-2-benzothiazolinone hydrazone (MBTH, usually used as hydrochloride monohydrate) with oxygen peroxide, and second, coupling of the oxidized MBTH with 3-(dimethylamino) benzoic acid (DMAB) resulting in the formation of the deep-purple indamine dye. The reactions involved in this assay are shown in Scheme III. In the UV region MBTH has maxima at $\lambda_1 = 266$ nm and $\lambda_2 = 300$ nm ($\varepsilon(266) = 8\,640$ L/(mol·cm) and $\varepsilon(300) = 5\,300$ L/(mol·cm)), DMAB has maximum at $\lambda = 306$ nm ($\varepsilon(306) = 1\,360$ L/(mol·cm)). For $\lambda > 400$ nm absorption of solutions with [MBTH] $< 10^{-4}$ mol/L and [DMAB] $< 10^{-3}$ mol/L are negligible. The indamine dye has the broad absorption in the region from 550 nm to 650 nm with maximum at $\lambda = 590$ nm. It has been found that by using the MBTH-DMAB-H_2O_2 system it is possible to determine HRP in the picomolar amounts either by the rate method (calibration based on the rate with which the absorption at 590 nm increases) or by the fixed time method (calibration based on the absorption at 590 nm developed after a given reaction time).

For measurements, we prepared the stock solutions of MBTH ([MBTH] = $2.79 \cdot 10^{-4}$ mol/L) and DMAB ([DMAB]) = $4.08 \cdot 10^{-3}$ mol/L) in PBS (pH = 7.4). Hydrogen peroxide was added to the solution of DMAB (2 μL of 30% H_2O_2 to 10 mL of the DMAB stock solution) just before the determination of HRP.

Determination of the activity of HRP was performed by the addition of 0.5 mL of the stock solution of MBTH and 10 μL of the solution of HRP, and/or of the suspension of the latex particles with attached HRP (samples containing from 0.15 to 1.5 pmole of HRP), to the 2.5 mL of the stock solution of DMAB and H_2O_2. Mixtures were incubated at 30 °C and exactly after 30 min the optical density at 590 nm (OD(590)) was registered. In the case of HRP attached to the latex the UV spectra were corrected for the contribution resulting from the light scattering from latex particles according to the method described earlier [31, 32].



Scheme III

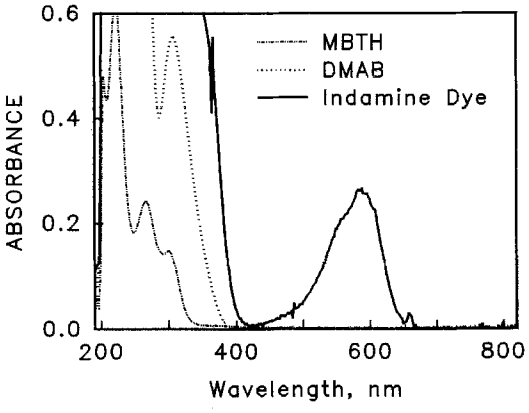


Fig. 1 The UV spectra of MBTH ([MBTH] = $2.79 \cdot 10^{-5}$ mol/L), DMAB ([DMAB] = $4.08 \cdot 10^{-3}$ mol/L) and of the indamine dye produced after 30 min of reaction involving MBTH ([MBTH] = $4.65 \cdot 10^{-5}$ mol/L), DMAB ([DMAB] = $3.40 \cdot 10^{-3}$ mol/L) HRP ([HRP] = $1.34 \cdot 10^{-10}$ mol/L), and H_2O_2 ([H_2O_2] = $1.47 \cdot 10^{-6}$ mol/L). Solvent PBS (pH = 7.4), 1 cm cell

The UV spectra of MBTH, DMAB, and of the indamine dye produced in reactions involving HRP are given in Fig. 1.

Determination of the surface concentration of HRP and oxidized HRP attached onto the latex particles

The amount of HRP and/or of HRP-OX attached to the latex particles was calculated from the difference of the protein content in solution (before incubation with latex) and in the supernatant (after removal of latex particles by centrifugation) or directly, for the isolated latex with attached protein, by using the modified Lowry method [31, 32]. Concentrations of proteins in solution were determined by the standard Lowry [33] and/or Bradford [34] methods. Both these methods are based on chemical reactions, involving proteins, leading to products with high extinction coefficients in the visible region. Concentrations of these products, easily determined by UV spectroscopy, are proportional to the protein concentrations. Determination of protein according to the Lowry method consists of two steps. In the first one the complex protein-Cu^{+2} is formed in the basic conditions. In the second step this complex is involved in the red-ox reactions with the mixture of the phosphomolybdic and phosphotungstic acids. Molybdenum and tungsten oxides, formed in these reactions in concentration proportional to the concentration of protein, are determined due to their absorption with maximum at 750 nm. Determination of proteins by the Bradford method is based on reaction between protein and the dye Coomassie Brilliant Blue G (BBG). Without protein BBG is present in the form of ion pairs absorbing light at $\lambda = 465$ nm. In reaction with protein (polycation in acidic conditions) BBG dissociates. Anions of BBG absorb visible light at $\lambda = 595$ nm. Concentration of BBG anions is proportional to the concentration of protein.

In each experiment, we used the known amounts of latex particles with the well determined averaged particle diameter and specific surface. The surface concentrations of attached protein was calculated as the ratio of the amount of protein attached to the latex and the surface of latex particles used for protein attachment.

All spectroscopic measurements needed for the determination of the amount and activity of free (in solution) and attached to latex HRP and oxidized HRP were made using the Hewlett Packard 8452A Diode Array UV spectrophotometer.

Results and discussion

Adsorption and covalent immobilization of HRP and HRP-OX to the P(SA), P(SA)-M, and P(A)-M latexes

Adsorption of HRP to the P(S) latex was carried in PBS (pH = 7.4). In each experiment the concentration of latex particles was equal to 3.1 mg/mL. The concentration of HRP was varied form 10 μg/mL to 250 μg/mL. Latex and known amounts of HRP were incubated at room temperature. The amount of adsorbed HRP was determined after 20 h of incubation. The adsorption isotherm, shown in Fig. 2, indicates that for the protein concentration in solution exceeding 0.05 mg/mL the surface concentration of

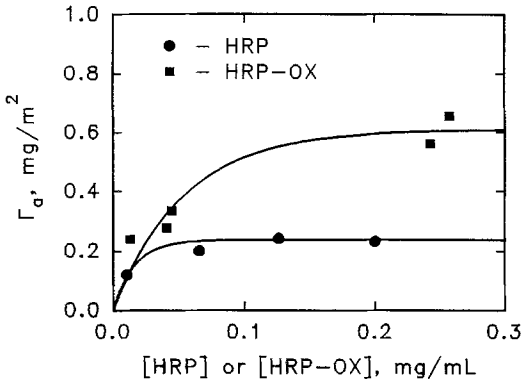


Fig. 2 Adsorption isotherms for HRP and HRP-OX on the P(S) latex. Conditions: [P(S)] = 3.1 mg/mL, PBS (pH = 7.4), temperature 20 °C, incubation time 20 h

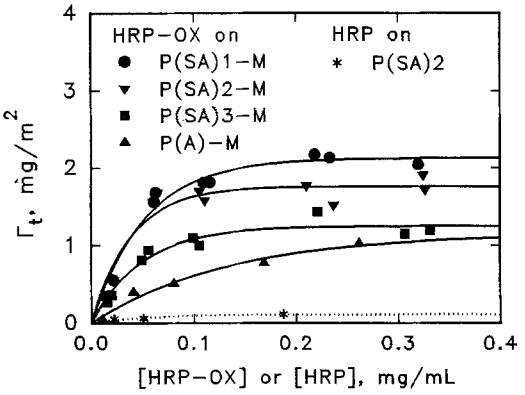


Fig. 3 Dependencies of the total surface concentrations (Γ_t) of HRP and HRP-OX, attached to latex particles, on the protein concentrations during immobilization. Conditions: [Latex] = 3.1 mg/mL, PBS (pH = 7.4), temperature 20 °C, incubation time 20 h

HRP reaches plateau equal to 0.24 mg/m^2, indicating saturation of the surface of P(S) latex with the adsorbed HRP macromolecules. Similar experiments were performed also for the HRP-OX. It is worth to note that in the case of HRP-OX the adsorption isotherm (cf. Fig. 2) indicates the significantly higher surface concentration corresponding to saturation than for the parent non-oxidized protein (0.65 mg/m^2 and 0.24 mg/m^2 respectively).

HRP and HRP-OX do not desorb spontaneously when P(S) latex particles with adsorbed protein were kept in pure PBS for several days. Such quasi-irreversible adsorption was observed earlier for several systems [26, 35, 36] and was attributed to the multipoint, mainly hydrophobic, protein-polymer interactions. However, when the P(S) particles with adsorbed HRP and/or HRP-OX were transferred to the 3% solution of sodium dodecylsulfate (SDS) in water the whole protein desorbed within 2 h.

Immobilization of HRP and HRP-OX onto the P(SA) and modified P(SA) and P(A) latexes (P(SA)-M latexes with amino groups) were performed in the same manner as adsorption onto the P(S) latex. The dependencies of the total (adsorbed and immobilized covalently) surface concentration of attached proteins (Γ_t) on the protein concentrations in solutions are shown in Fig. 3. Plots collected in Fig. 3 indicate that the surface concentrations of HRP attached to the P(SA) 2 latex are several times lower than for the HRP-OX attached to the modified P(A) (P(A)-M) latex and to any of the P(SA)-M latexes. This large difference between the efficiency of the attachment of HRP-OX to latexes with amino groups and the attachment of HRP to the P(SA) 2 latex with aldehyde groups may look strange, however, it conforms to the opinion that in HRP supplied by companies selling chemicals for the laboratory and diagnostic purposes the significant portion of the amino groups is blocked with isothiocyanate and thus, cannot participate in the immobilization reaction with aldehyde groups [29, 37]. Modification of latex particles, resulting in the introduction of amino groups and oxidation of HRP, producing aldehyde groups in the carbohydrate portion of the enzyme, opens the way for its efficient covalent immobilization.

Treatment of P(SA)-M latexes, containing HRP-OX on the surface, with 3% SDS for 2 h removed part of the attached enzyme. For HRP-OX on the P(S) latex, we noticed the complete protein desorption with SDS and for the P(A)-M latex a similar treatment with SDS left the whole amount of HRP-OX not detached from the surface. Thus, we could assume that in the case of P(SA)-M latexes SDS removes the fraction of adsorbed HSA-OX. The amount of liberated HRP-OX from the known amount of latex particles allowed to determine the surface concentration of adsorbed enzyme (Γ_a). The surface concentration of the covalently immobilized HRP-OX was calculated as the difference between the total attached and the adsorbed surface concentrations of the enzyme ($\Gamma_c = \Gamma_t - \Gamma_a$). Plot of Γ_c of HRP-OX immobilized covalently on the P(SA)-M latexes as function of the enzyme concentration in solution during immobilization is given in Fig. 4.

The dependencies of the maximal surface concentrations of the: total (Γ_t(max)), adsorbed (Γ_a(max)), and covalently immobilized (Γ_c(max)) HRP-OX on the fraction of polyacrolein in the surface layer (f_A) of the P(SA) latexes are shown in Fig.5. The maximal surface concentration of adsorbed HRP-OX is proportional to the fraction of polystyrene ($f_S = 1 - f_A$) in the surface layer. The relation between the surface concentration of the covalently immobilized HRP-OX and f_A indicates that for latexes with $f_A \geq 0.50$ the increasing fraction of polyacrolein leads to the lower values of Γ_c. The same was true for Γ_t. Assuming

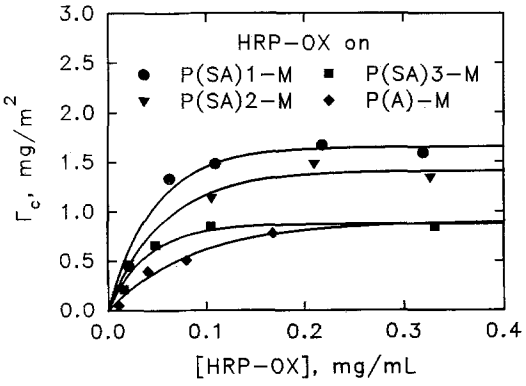


Fig. 4 Dependencies of the surface concentrations of HRP-OX, immobilized covalently on the P(SA)-M latexes, on the protein concentrations during immobilization. Conditions of immobilization: [Latex] = 3.1 mg/mL, PBS (pH = 7.4), temperature 20 °C, incubation time 20 h

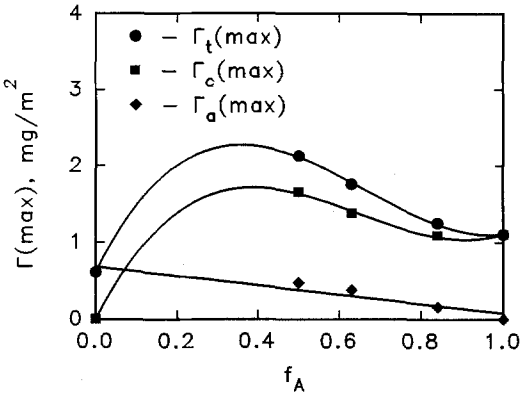


Fig. 5 Dependencies of the maximal surface concentrations of the total (Γ_t(max)), adsorbed (Γ_a(max)), and covalently immobilized (Γ_c(max)) HRP-OX on the fraction of polyacrolein in the surface layer of the P(SA)-M latexes

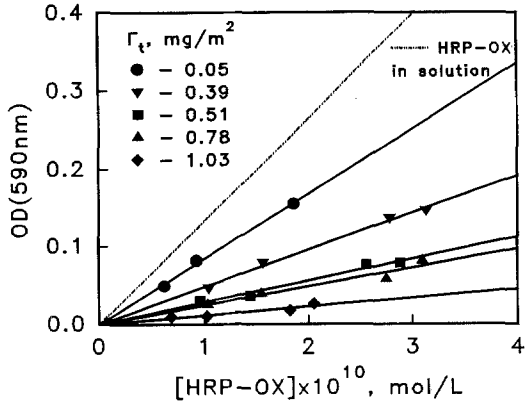
that all aldehyde groups on the surface of the P(SA) latex particles react with the amino groups of ethylenediamine (this has been shown recently for even bulkier 2,4-dinitrophenylhydrazine [38]), one can conclude that the increasing number of amino groups involved in the immobilization of HRP-OX results in more extensive enzyme denaturation requiring more space on the surface. Similar observations were made recently for the attachment of human serum albumin onto the P(SA) latexes [26].

Enzymatic activity of HRP and HRP-OX in solution and attached to the latex particles

The enzymatic activity of HRP and HRP-OX, in solution and attached to the latex particles, was determined by measuring the optical density (OD(590)) corresponding to formation of the indamine dye produced in the process involving enzymatic oxidation of MBTH catalyzed with peroxidase (cf. the experimental part). The determinations were made for various concentrations of enzymes. For enzymes attached to latex particles, regardless of the actual surface concentrations, the concentrations used for the plots of enzyme activity were calculated as the ratio of the number of moles of the attached enzyme and the volume of suspension containing latex particles. Plots of OD(590), measured for HRP-OX immobilized on the P(A)-M latex, are given in Fig. 6. Measurements were made for latexes with different surface concentrations of HRP-OX. Plots are represented by the straight lines crossing the origin of coordinates. Slopes of these lines, describing OD(590) corresponding to the unit concentration of an enzyme, characterize the specific enzyme activity (Ac = Δ OD(590)/Δ [HRP-OX]) of HRP-OX on the given latex. Plot for HRP-OX in PBS is also given for comparison. Similar measurements were performed also for HRP-OX on the P(S), P(SA) 1-M, P(SA) 2-M, and P(SA) 3-M latexes and for HRP on the P(S) latex. The activity of HRP on P(SA) 1, P(SA) 2, P(SA) 3, and P(A) latexes could not be determined because the surface concentrations of the attached enzymes was too low (< 0.05 mg/m^2) to allow any reliable measurements. Plots of the specific activity (Ac) of enzymes attached to the latex particles are given in Figs. 7 and 8. In these figures are shown also the specific activities of HRP and HRP-OX in solution. The specific activities of HRP and HRP-OX corresponding to the saturation of the surface of latex particles with enzyme macromolecules are given in Table 2.

Measurements of activity of HRP and HRP-OX indicate that oxidation of the carbohydrate fragment of HRP leads to the noticeable reduction of the enzyme activity.

Fig. 6 Absorption corresponding to the indamine dye (OD(590)) produced in the enzymatic reaction catalyzed with HRP-OX in solution and/or immobilized covalently on the P(A)-M latex. Latexes with various surface concentrations of HRP-OX were used



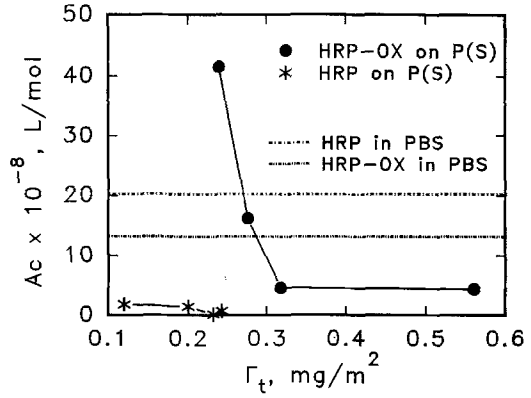


Fig. 7 Specific activity (Ac) of HRP and HRP-OX adsorbed onto P(S) latex for protein-latex systems with various values of Γ_a. Latexes suspended in PBS (pH = 7.4). Specific activities of HRP and HRP-OX are indicated for the comparison

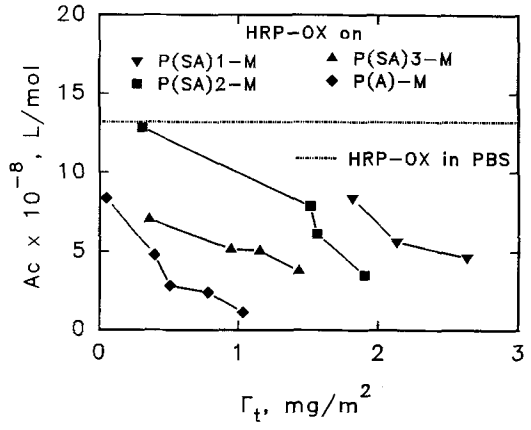


Fig. 8 Specific activity (Ac) of HRP-OX attached to P(SA) 1-M, P(SA) 2-M, P(SA) 3-M, and P(A)-M latexes for protein-latex systems with various values of Γ_t. Latexes suspended in PBS (pH = 7.4). Specific activity of HRP-OX in PBS is shown for the comparison

The specific enzymatic activity equals $2.02 \cdot 10^9$ L/mol and $1.32 \cdot 10^9$ L/mol for HRP and HRP-OX respectively. The reduction of the activity of HRP upon oxidation (activity from 56 to 94% of original depending on the oxidation conditions) was reported also in the earlier studies [29], however, it is not clear which step of the oxidation process is responsible for it.

The adsorption of HRP onto the P(S) latex results in the almost complete loss of the enzyme activity (cf. Fig. 7). The inactivation of HRP on the low energy surfaces was observed earlier for poly(ethylene) [17] and for poly(tetrafluoroethylene) [39]. HRP adsorbed onto the higher energy surfaces (e.g., poly(ethyleneterephthalate) and tetrachloroethylene glow discharge treated poly(ethyleneterephthalate)) retains more of its enzymatic activity [39].

Table 2 Specific activity (Ac) of HRP-OX corresponding to the maximal coverage (saturation) of latex particles.

Latex	Fraction of polyacrolein in the surface layer of latex particles	Fraction of HRP-OX immobilized covalently $\Gamma_c(max)/\Gamma_t(max)$	Specific activity of HRP-OX attached to the latex particles $AC \cdot 10^{-8}$ L/mol
P(S)	0	0	4.36
P(SA)1-M	0.50	0.77	4.62
P(SA)2-M	0.63	0.78	3.50
P(SA)3-M	0.84	0.87	3.70
P(A)-M	1.00	1.00	1.12

The behavior of HRP-OX is different from that of the parent HRP. Namely, the specific activity of HRP-OX was strongly dependent on the surface concentration of adsorbed protein. For the low surface concentrations it exceeded the specific activity of HRP-OX (for $\Gamma_a < 0.3$ mg/m^2) and even HRP (for $\Gamma_a < 0.25$ mg/m^2). Apparently, the oxidation process involving blocking of the amino groups in HRP with 2,4-dinitroflurobenzene and oxidation of the carbohydrate portion of HRP result in more rigid structure of the enzyme, reducing its denaturation during adsorption and, in effect, preventing the deactivation. The increasing activity of HRP-OX following its conjugation with immunoglobulins was observed by Nakane and Kawaoi [29]. Presumably, the adsorption onto the P(S) latex and conjugation of HRP-OX with immunoglobulins change the local environment of the enzyme macromolecules (e.g. pH and ionic strength within the double layer of the latex particles) in the way increasing its activity.

The specific activity of HRP-OX attached to the P(S), P(SA)-M, and P(A)-M latexes decreased with the increasing surface concentration of the enzyme. Apparently, the dense coverage of the surface of latex particles either results in the partial blocking of the active centers or, due to the cooperative interactions involving closely located HRP-OX macromolecules, the release of the product of enzymatic oxidation is slowed down.

Data collected in Table 2 illustrate the dependence of the specific activity of HRP-OX on the latex particles (for the maximal coverage of the latex surface with the enzyme macromolecules) on the fraction of enzyme immobilized covalently. The increased fraction of HRP-OX immobilized covalently results in the lower specific activity of attached HRP-OX macromolecules. This observation, like the relation between the fraction of polyacrolein in the surface layer and maximal surface concentration of attached HRP-OX discussed in the previous section, indicate that the increasing covalent immobilization results in more extensive denaturation of enzyme macromolecules

and in the loss of their activity. However, it is worth noting that, for the properly chosen latex, (particles with fraction of polyacrolein in the surface layer from 0.50 and 0.85), the procedure describe in this paper allows to obtain systems with high fraction of the covalently immobilized enzyme (exceeding 0.75) with the specific activity from 19% to 23% of that of HRP and from 29% and 35% of the specific activity of HRP-OX.

Conclusions

- Polystyrene latex obtained in emulsifier-free polymerization can be used for adsorption of HRP, however, upon adsorption the HRP macromolecules lose their enzymatic activity.
- Incubation of HRP with poly(styrene/acrolein) latexes does not lead to the adsorption and/or covalent immobilization of the enzyme macromolecules.
- Blocking of amino groups in HRP with 2,4-dinitrophenylfluorobenzene and oxidation of the carbohydrate portion of HRP with sodium periodate lead to HRP-OX with reduced (65% specific activity). However, the specific activity of HRP-OX could be increased upon adsorption onto the polystyrene latex.
- The modification of the surface of latex particles, with blocking of the surface aldehyde groups and introduction of the amino groups, provides the polymeric support suitable for the controlled adsorption and covalent immobilization of HRP-OX.
- The HRP-OX attached to the poly(styrene/acrolein) latexes (fraction of the covalently immobilized enzyme exceeding 75%) retains ca. 30% of its activity before immobilization.

References

1. Rembaum A, Yen SPS, Molday RS (1979) J Macromol Sci, Chem A13:603
2. Guiot P, Couvreur P (eds) (1986) Polymeric Nanoparticles and Microspheres. CRC, Boca Raton, FL
3. Rembaum A, Tökes ZA (eds) (1988) Microspheres: Medical and Biological Applications. CRC Press, Boca Raton, FL
4. Ugelstad J, Berge A, Ellingsen T, Schmid R, Nilsen T-N, Mørk PC, Stenstad P, Hornes E, Olsvik O (1992) Prog Colloid Polym Sci 17:87
5. Polysciences Inc, Data Sheet No 238 (1984) with over 70 references to original papers discussing applications of latexes and microspheres in medical diagnostics
6. Colowick SP, Kaplan NO (eds) (1987) Immobilized Enzymes and Cells, Part C, vol 136 In: Mosbach K (series ed) Methods in Enzymology, Academic Press, Orlando
7. Marumoto K, Suzuta T, Noguchi H, Uchida Y (1978) Polymer 19:867
8. Goers JWF (1993) In: Immunochemical Techniques; Laboratory Manual. Academic Press, San Diego, CA, p 137
9. Marcus L, Offarim S, Margel S (1982) Biomat, Med Dev, Art Org 10:157
10. Rembaum A, Yen RCK, Kempner DH, Ugelstad J (1982) J Immun Meth 52:341
11. Margel S (1984) J Polym Sci, Polym Chem Ed 22:3521
12. Rembaum A, Chang M, Richards G, Li M (1984) J Polym Sci, Polym Chem Ed 22:609
13. Colvin M, Smolka A, Chang M, Rembaum A (1988) In: Ref. [3] p 1
14. Yoshida M, Asano M, Yokota T, Chosdu R, Kumakura K (1989) J Polym Sci; Part C; Polym Lett 27:437
15. Yoshida M, Asano M, Yokota T, Kumakura M (1990) Polymer 31:371
16. Gabel D, Steinberg IZ, Katchalsky E (1971) Biochemistry 10:4461
17. Sandwick RK, Schray KJ (1987) J Colloid Interface Sci 115:130
18. Pye EK, Chance B (1976) In: Mosbach K (ed) Immobilized Enzymes, vol 44 in Methods in Enzymology. Academic Press, New York, p 111
19. Chang M, Colvin M, Rembaum A (1986) J Polym Sci Polym Letters Ed 24:603
20. Chen JP, Kiaei D, Hoffman AS (1993) J Biomater Sci Polymer Edn 5:167
21. Malmatadt HV, Hadjioannou TP (1963) Anal Chem 35:14
22. Greewalt TJ, Swierk EM, Steane EA (1975) J Immunol Methods 8:351
23. Ref [8] p 119
24. Margel S, Beitler U, Offarim M (1982) J Cell Sci 56:157
25. Basinska T, Slomkowski S, Delamar M (1993) J Bioact Compat Polym 8:205
26. Basinska T, Slomkowski S (1993) In: Guilbault G, Mascini M (eds) Uses of Immobilized Biological Compounds. Kluwer Academic Publishers, Dordrecht, Boston, London, p 453
27. Basinska T, Kowalczyk D, Slomkowski S, Wang FW (1994) In: Schmitz KS (ed) Macro-ion Characterization From Dilute Solutions to Complex Fluids, ACS Symp Ser 548. ACS, Washington, DC, p 449
28. Miksa B, Slomkowski S (1993) In: Kahovec J (ed) Macromolecules 1992. VSP International Science Publishers, p 15
29. Nakane PK, Kawaoi A (1974) J Histochem Cytochem 22:1084
30. Ngo TT, Lenhoff HM (1980) Anal Biochem 105:389
31. Basinska T, Slomkowski S (1991) J Biomater Sci Polymer Edn 3:115
32. Basinska T, Slomkowski S (1992) In: Daniels ES, Sudol ED, El-Aasser MS (eds), Polymer Latexes, Preparation, Characterization, and Applications, ACS Symp Ser 492 p 328
33. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) J Biochem 193:265
34. Bradford MM (1976) Anal Biochem 72:248
35. Andrade JD, Hlady V (1986) Adv Polym Sci 79:1
36. Norde V, Lyklema J (1991) J Biomater Sci Polymer Edn 3:183
37. Nakane PK, Ram JS, Pierce GB (1966) J Histochem Cytochem 14:789
38. Miksa B, Slomkowski S (1995) J Biomater Sci Polymer Edn 273:47
39. Chen JP, Kiaei D, Hoffman AS (1993) J Biomater Sci Polymer Edn 5:167