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ORIGINAL CONTRIBUTION

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Polypyrrole core/polyacrolein shell latex for protein immobilization

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Abstract The redox polymerization of pyrrole, with ferric chloride as oxidant, carried out in the presence of polyvinylpyrrolidone (PVP), yielded polypyrrole latex particles. The polypyrrole latex was used, as seed, for the radical polymerization of acrolein. The resulting polypyrrole core/polyacrolein shell latex (poly(P/A)) was suitable for immobilization of up to 11 mg of human serum albumin (HSA) and/or 33 mg of human gamma globulin (γG) per 1 g of latex particles.

Key words polypyrrole – polyacrolein – core-shell latex – protein immobilization

Introduction

Latexes, suitable for immobilization of proteins, are often used as important elements of various diagnostic systems [1–3]. The basic requirements which the prospective latexes for diagnostic applications have to fulfill include their ability to adsorb and/or covalently immobilize proteins (usually immunoglobulins) and the convenience of observation in suspension and/or as precipitates on solid surfaces. The development of synthetic methods leading to the monodisperse polystyrene latexes [4] and to the polystyrene latexes equipped with various functional groups for covalent immobilization of proteins [5–8], facilitated application of these particles for the diagnostic assays. The large number of latexes for diagnostic systems has been developed also on the basis of acrylic monomers [9–11]. However, polystyrene and common polyacrylate polymers do not absorb light in the visible region and without additional incorporation of pigments, and/or chromophore labels the direct observation of latex suspensions has to rely solely on the light scattering on these particles.

Studies on the polymerization of pyrrole led to the new material which is potentially very useful for the diagnostic applications. The doped polypyrrole, obtained in the redox and/or electrochemical polymerization, is known to be deeply black. This property, as well as the relatively high electrical conductivity of this polymer, are presumably due to the cation-radical nature and the extensive charge delocalization in the polypyrrole macromolecules [12, 13]. Thus, particles of polypyrrole have been considered to be good candidates for solid supports for diagnostic purposes, especially for the so-called "dry diagnostic tests" in which the only liquid reagent is the analyzed substance and presence of particles in a given place on the special blotting paper has to be monitored with the naked eye [14]. In such applications the polypyrrole latex can be observed as easily as an Indian ink.

It has been known that the redox polymerization of pyrrole, carried out in the presence of the surface active

agents (poly(vinyl alcohol-co-vinyl acetate), poly(vinyl acetate), poly(*N*-vinylpyrrolidone), and poly(ethylene oxide)), leads to polypyrrole in the form of latexes with particle diameters ranging from ca 50 to 300 mm, depending on the polymerization conditions [15–18]. Unfortunately, the attachment of proteins to the surface of these particles is very inefficient, which, in this form, makes the polypyrrole latex unsuitable for diagnostic purposes. Recently, Tarcha et al. [14] described the derivatization procedures introducing bromoacetyl, carboxylate and/or amino groups onto the surface of the polypyrrole latex. According to this method polypyrrole latex was acetylated in *N*-methyl pyrrolidone by using bromoacetyl bromide. The bromoacetylated latex could be converted into latexes with carboxylic and or amino groups in reaction with thioacetic acid and triethylene tetramine. Latexes obtained in this way were suitable for covalent immobilization of proteins and, eventually, for diagnostic applications [14]. The methods of functionalization, mentioned above, are rather complex and inconvenient. The parent polypyrrole latexes were obtained in water, bromoacetylation had to be carried out in the anhydrous conditions, immobilization of proteins had to be carried out in buffers. Thus, on the way from the primary polypyrrole latex to the latex suitable for the immobilization of proteins, it was necessary to replace the reaction medium two times (from water to anhydrous *N*-methyl pyrrolidone and again to water solutions) and at certain stages to redisperse the aggregated latexes by sonication.

Some time ago Rembaum et al. [5, 10, 19, 20], Marcus [21] et al. and Margel [22] described synthesis of polyacrolein and polyacrolein containing microspheres for the covalent immobilization of proteins. Recently, we investigated immobilization of proteins onto the polystyrene latexes containing various fractions of polyacrolein, providing aldehyde groups for the direct immobilization of protein in the surface layer [7, 23, 24]. It has been found also that polyacrolein could be grafted onto polystyrene latex by polymerization of acrolein in the presence of the latex seed [5].

In this paper we describe the synthesis of polypyrrole core/polyacrolein shell latex, by the polymerization of acrolein, carried out in the water medium in presence of the earlier obtained polypyrrole latex. Attachment of human serum albumin (HSA) and human gamma globulins (γG) to the pure polypyrrole latex and to the polypyrrole core/polyacrolein shell latexes are also compared.

Experimental part

Polypyrrole latex (poly(P)) was synthesized in a manner similar to that described by Armes et al. [15]. A 0.8 g sample of poly(*N*-vinylpyrrolidone) (Aldrich, K30) was dissolved in 200 mL of water (distilled three times). This solution was placed into a three-nacked, round bottom flask equipped with a stirrer. Subsequently, 35.3 g of $FeCl_3 \cdot 6H_2O$ in 200 mL of water and 4 mL of freshly distilled pyrrole (Aldrich) were added to the flask. The reaction was carried out during 24 h under N_2, at room temperature, with stirring (60 rpm). The synthesized latex was isolated by centrifugation (30 min at 9000 G) and redispersed in the fresh portion of water. The procedure, mentioned above, was repeated two more times. After the last redispersion, we obtained 40 mL of latex suspension containing 6.44 wt/vol % of the polypyrrole latex.

Polypyrrole core/polyacrolein shell latex (poly(P/A)1) was obtained by the radical polymerization of acrolein initiated with $K_2S_2O_8$ and carried out in the presence of poly(P). Suspension of poly(P) (50 mL), containing 0.6 g of latex particles and 0.022 g of $K_2S_2O_8$, was placed in the round bottom flask. After purging, the suspension with N_2 0.1 mL of freshly distilled acrolein (Aldrich) was added to this mixture. Polymerization was carried out at 65 °C with stirring (60 rpm) for 30 h. The synthesized latex particles were purified by the four-times repeated centrifugation (for 30 min at 5 000 G) and redispersion in water (distilled three times). Latex designated further as poly(P/A)2 was obtained in a similar manner but for synthesis the two times higher amount of polyacrolein has been used (0.2 mL of acrolein for 0.6 g of latex in 50 mL of water).

Human serum albumin (HSA) (Sigma, Cohn fraction V) and human gamma globulins (γG) (Sigma, Cohn fractions II and III) were used as received.

Immobilization of HSA and/or γG was carried out by gentle mixing protein solution in the phosphate buffer saline (PBS, pH = 7.4) with latex in water suspensions. The amount of immobilized protein was evaluated form the protein concentration in solution (before incubation with latex) and in the supernatant (after incubation followed with latex isolation by centrifugation). Protein concentrations were determined spectrophotometrically by measuring adsorption at 280 nm.

Scanning electron micrographs were registered by using a Hitachi S-570 apparatus.

Diameters of latex particles were measured using a BI-90 Particle Sizer V3.9 (Brookhaven Instruments Corporation).

Surface concentration of the cationic groups on latex particles was determined by conductometric titration with 0.001 n HCl. Prior to the titration latexes were passed through the Amberlit IRA400 exchange resin.

UV spectra were registered using a Hewlett Packard 4852A diode array spectrophotometer.

Results and discussion

The diameter distributions of poly(P), poly(P/A)1, and poly(P/A)2 latexes are given in Fig. 1. The SEM micrograph of poly(P) latex is shown in Fig. 2. From the plots given in Figure 1 it is evident that the poly(P/A)1 and poly(P/A)2 latexes, obtained by polymerization of acrolein in the presence of poly(P) latex, are monomodal. Moreover, the diameters of poly(P/A)1 and poly(P/A)2 particles are larger than for the parent poly(P) latex (cf. Table 1). This observation conforms to the hypothesis that during polymerization of acrolein, carried out in the presence of poly(P) latex, the precipitating polyacrolein does not form separate particles but becomes attached to the poly(P) seeds. Presumably, the positive charge of poly(P) latex (polypyrrole in latex particles obtained by the redox polymerization is positively charged [13–16]) facilitates attachment of polyacrolein macromolecules which are equipped, due to the initiation with radical anions from $K_2S_2O_8$, with the negatively charged sulfate end groups.

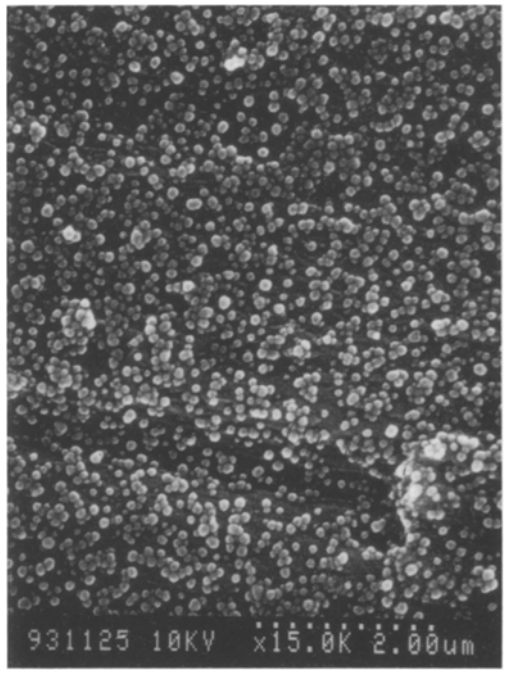

Fig. 2 SEM microphotogram of the poly(P) latex

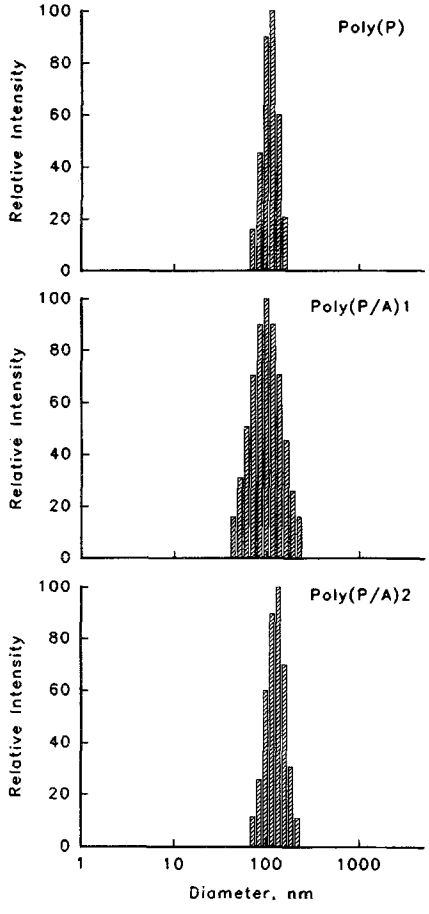

Fig. 1 Diameter distributions of poly(P), poly(P/A)1, and poly(P/A)2 latexes

The attachment of polyacrolein macromolecules with anionic end-groups to the polypyrrole latex seeds was manifested by the reduction of the positive surface charge of the latex particles. The surface charge, determined by the conductometric titration, was equal to $9.4 \cdot 10^{-7}$ mol/m^2 ($4.46 \cdot 10^{-5}$ mol/g) for poly(P) seed particles, $7.1 \cdot 10^{-7}$ mol/m^2 ($2.23 \cdot 10^{-5}$ mol/g) and $4.7 \cdot 10^{-7}$ mol/m^2 ($1.35 \cdot 10^{-5}$) for poly(P/A)1 and poly(P/A)2 respectively. In these calculations we assumed that the density of poly(P), poly(P/A)1, and poly(P/A)2 is the same for the aminated polypyrrole latex and equal 1.36 g/cm^3 [14]. It is worth to note that the progressing deposition of the polyacrolein macromolecules, with anionic end-groups, on the cationic polypyrrole particles may lead, at certain stage, to the loss of charge stabilization. Indeed, in the polymerization of 0.6 g of acrolein initiated with 0.025 g of $K_2S_2O_8$ and carried out in the presence of 0.6 g of polypyrrole latex particles we noticed latex aggregation for conversion of acrolein exceeding ca 70%.

Various methods are described in the literature for determination of the aldehyde groups on the polyacrolein containing latexes. However, one has to be aware that none of them can be considered as ideal. For example, Rembaumn et al. [19] and Margel [22] used hydroxylamine for the covalent immobilization involving aldehyde groups of latex particles. Determination of nitrogen in particles treated with this reagent was used as the basis for evaluation of aldehyde groups on microspheres.

Table 1 Parameters characterizing the polypyrrole and polypyrrole core/polyacrelein shell latex

Latex	Intensity Averaged Size mm	Standard Deviation nm	Mass Median Diameter[a] (MMD) mm	Geometric Standard Deviation[a] (GSU)	Surface Concentration of -CHO Groups[b] mol/g	Surface Concentration of Cationic Groups mol/g
poly(P)	124	26	121	1.2	–	$3.56 \cdot 10^{-5}$
poly(P/A)1	153	65	141	1.5	$7.93 \cdot 10^{-6}$	$2.23 \cdot 10^{-5}$
poly(P/A)^2	154	56	148	1.3	$7.47 \cdot 10^{-6}$	$1.35 \cdot 10^{-5}$

[a]) The lognormal distribution by weight is given by:

$$dW = (1/(\ln(\mathrm{GSU})\sqrt{2\pi})\exp(-(\ln(D) - \ln(\mathrm{MMD}))^2/(\ln(\mathrm{GSU})\sqrt{2})^2)d(\ln(\mathrm{D}))$$

where dW denotes fraction of particles with diameters between $\ln(D)$ and $\ln(D) + d(\ln(D))$.
[b]) Concentration of aldehyde groups in the surface layer of latex particles which are accessible for 1-aminopyrene.

For polyacrolein microspheres with diameter 0.1 μm, Margel found, in this way, from 2.9 to 12 mmol of aldehyde groups per 1 g of particles, depending on the actual method of their synthesis [20]. Calculations indicate that for polyacrolein chains with ideal structure $-CH_2-CH(CO)-$ the above-mentioned content of aldehyde groups corresponds to penetration of hydroxylamine from 30 to 160 Å deep into the particle (i.e., the thickness of the shell penetrated with hydroxylamine ranges from ca. 6 to 30% of the particle radius). Later, it was found [6] that hydroxylamine is able to penetrate significantly also the surface layer of the pure polystyrene particles. Thus, methods based on this reagent provide highly overestimated values of the surface concentration aldehyde groups on latex particles.

XPS Spectroscopy, used for determination of the surface content of comonomer with aldehyde groups on poly(styrene/*p*-formylstyrene) [6] and on poly(styrene/acrolein) [7] latex particles, also provides information on the composition of the surface layer not less than ca. 50 Å thick. We investigated labeling of the surface of polystyrene, poly(styrene/acrolein) and polyacrolein latexes with 2,4-dinitrophenylhydrazine (DNPH) [7]. These studies indicated that DNPH leaves the pure polystyrene particles unlabeled, but labels the surface layer of the poly(styrene/acrolein) particles to the depth ca from 1.5 to 3.5 Å (assuming that the thickness of the layer analyzed by XPS is equal to 50 Å).

The hydroxylamine method was inconvenient for determination of aldehyde groups on the surface of poly(P/A) latex particles, not only due to the deep penetration of this amine inside latex particles, but also because of the high nitrogen content in the polypyrrole core masking the nitrogen introduced during reaction with hydroxylamine. We found also that DNPH cannot be used in the case of poly(P/A) latexes because it also labels the poly(P) latex.

Recently, we compared labeling of the poly(styrene/acrolein) latexes with 1-aminopyrene (APY) and DNPH [24]. For APY the efficiency of immobilization was ca. 50% lower than for one much less bulky DNPH. However, we noticed that the surface concentration of immobilized APY increased in parallel with the change of polyacrolein in the surface layer determined by XPS. Thus, we decided to use the depletion of (APY) from ethanol solution (for APY $\varepsilon_{358} = 15\,800\,\mathrm{L/(mol.cm)}$), after incubation with known amount of latex particles, to estimate the amount of aldehyde groups on their surface. However, one has to be aware that in any measurements based on the label–surface interaction the results characterize not only the surface but the labels as well.

For the poly(P/A)1 and poly(P/A)2 latexes, we found the surface concentration of aldehyde groups accessible for APY to be $\approx 8 \cdot 10^{-6}$ mol/g (this corresponds to $2 \cdot 10^{-7}$ mol/m^2), whereas for the pure polyacrolein and for the variety of the poly(styrene/acrolein) particles, obtained by the similar polymerization carried out in absence of polypyrrole, we found the concentration of aldehyde groups accessible for the DNPH labels to vary from $7 \cdot 10^{-7}$ mol/m^2 to $2.5 \cdot 10^{-6}$ mol/m^2, depending on the polyacrolein content in the surface layer [23, 24]. Thus, even taking into account the lower efficiency (ca 50%) for the immobilization of APY, in comparison with 2,4-dinitrophenylhydrazine, we conclude that the surface concentration of aldehyde groups for poly(P/A)1 and poly(P/A)2 is much lower than for the pure poly(acrolein) latexes obtained by using the same initiating system. Rembaum and Margel found that during polymerization of acrolein some fraction of aldehyde groups is involved in side reactions leading to ether linkages [19, 22]. It is possible that these side reactions are facilitated by the presence of the cationic polypyrrole latex.

The UV spectra of the water suspensions of poly(P), poly(P/A)1 and poly(P/A)2 latexes are shown in Fig. 3. It can be seen that, regardless whether the polypyrrole latexes are covered with polyacrolein layer or not, the absorptions of their suspensions in the ultraviolet, visible, and near infrared regions are very high. Absorptions of

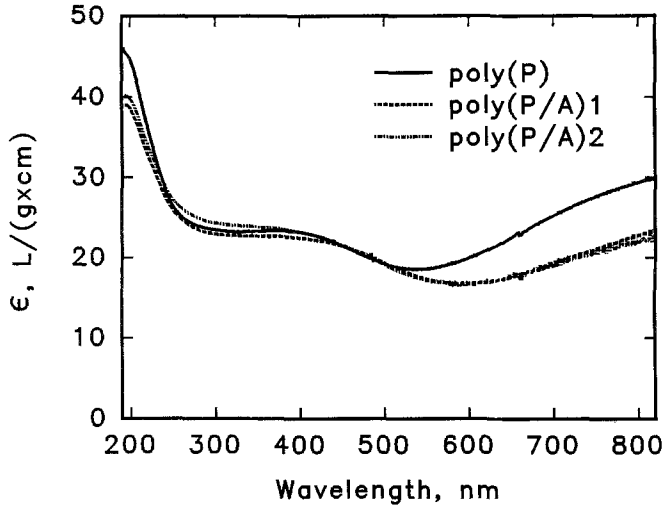

Fig. 3 UV spectra of poly(P), poly(P/A)1, and poly(P/A)2 latexes

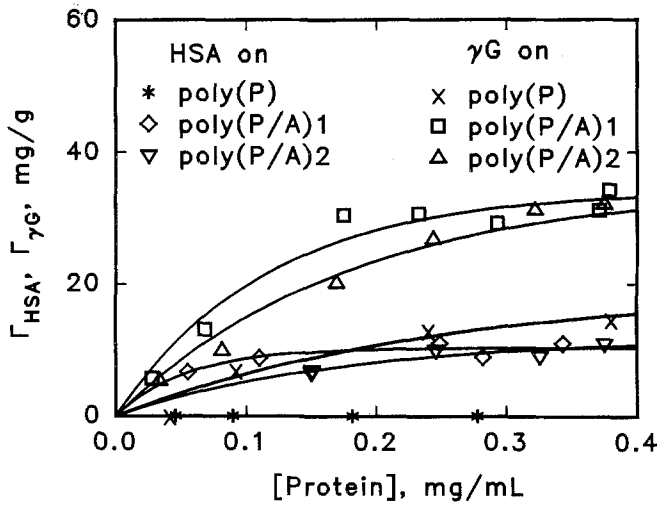

Fig. 4 Concentrations of HSA and γG attached to the surfaces of latex particles (in mg of protein per 1 g of latex particles) as function of the concentrations of proteins in solution. Concentration of latexes was equal to 3.1 ml/mL

latex suspensions obeyed Beer's law (at least for the latex concentrations lower than $5 \cdot 10^{-2}$ g/L) and at 550 nm (at or close to the minimum of absorption) the corresponding extinction coefficients were equal to 19.3, 19.4, and 19.2 $L \cdot g^{-1} \cdot cm^{-1}$ for poly(P), poly(P/A)1, and poly(P/A)2 latexes respectively.

It has been found earlier that the fractions of the HSA and/or γG physically adsorbed to the polyacrolein surface are negligible, if any, in comparison to the fractions of the corresponding proteins immobilized covalently [19, 22, 23]. The formation of Schiff base linkages between the amino groups of protein macromolecules and aldehyde groups of latex particles is reversible, however, the multivalent immobilization successfully eliminates spontaneous detachment of ligands with several primary amino groups [22]. Nevertheless, in the presence of free protein the exchange between protein macromolecules in the solution and on the surface of polystyrene core/polyacrolein shell latex particles was observed [25].

In all experiments with attachment of proteins to the poly(P) and poly(P/A) latexes the concentration of latex particle was equal to 3.1 mg/mL. Protein concentrations were varied from 25 to 400 μg/mL and the time of incubation, equal to 20 h, exceeded 2 h required for immobilization of protein onto the polyacrolein containing microspheres [19, 22]. The immobilization isotherms for HSA and γG are given in Figure 4 (we deliberately use this terminology remembering that until the Schiff base linkages became reduced, e.g. by the sodium cyanoborohydride, the immobilization cannot be considered as permanent and attached proteins may participate in slow exchange with other compounds containing primary amino groups). The dependencies of the concentrations of immobilized proteins (Γ_{HSA} and $\Gamma_{\gamma G}$) on the concentrations of protein in solution indicate that initially Γ_{HSA} and $\Gamma_{\gamma G}$ increase with increasing concentration of protein in solution until the plateau is reached that corresponds to the complete coverage of the surface of latex particles with protein macromolecules. According to the plots given in Fig. 4, HSA was not attached to the poly(P) latex, whereas the maximal concentrations of this protein, which could be attached to the poly(P/A)1 and poly(P/A)2 latexes are similar. For poly(P/A)1 and poly(P/A)2 Γ_{HSA} (max) are equal to 10 and 11 mg of protein attached to 1 g of the corresponding latex particles. The maximal concentration of γG attached to the unmodified poly(P), equal to $\Gamma_{\gamma G}(max) = 16$ mg/g, was also significantly lower than for the polypyrrole core/polyacrolein shell latexes. $\Gamma_{\gamma G}(max)$ was equal to 33 and 31 mg/g for poly(P/A)1 and poly(P/A)2, respectively.

It is interesting to compare the ability of poly(P/A) and polyacrolein latexes to immobilize γG. According to Margel, 1 g of the polyacrolein microspheres with 0.1 μm diameter is able to bind ca 60 mg of rabbit γG, which is equal to the surface density of immobilized protein 1 mg/m² [22]. The maximal surface density of attached human γG onto the poly(P/A)1 and poly(P/A)2 latex particles, determined in our studies, was equal to 0.8 mg/m².

Gamma globulins are composed mainly from IgG. Making the simplifying assumption that γG contains only IgG, we could estimate that the number of immobilized macromolecules per 1 latex particle can be varied in the controlled way from 0 to 80, to 240, and to 270 for poly(P), poly(P/A)1, and poly(P/A)2, respectively. In the case of the polypyrrole latex modified as described by Tarcha et al. [14] the number of immobilized antibodies per one latex

particle was close to 20. It is worth to note that the maximal number of IgG macromolecules which could be attached to one latex particle with diameter 119 nm, calculated by these authors, was equal to 285. Thus, taking into account that the polypyrrole core/polyacrolein shell latex described in this paper had similar diameters, we could conclude that the procedure described in this paper allows to synthesize polymeric supports suitable for the controlled immobilization of proteins from 0 to the complete coverage of the polypyrrole core latexes.

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