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Monodisperse carboxylated polystyrene particles: synthesis, electrokinetic and adsorptive properties

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Abstract

Effect of conditions of styrene dispersion polymerization initiated by 4,4′-azo-*bis*-(4-cyanopentanoic acid) in ethanol solutions of polyvinylpyrrolidone was investigated. Suitable methods ensuring the control of final particle size, surface structure, and surface concentration of carboxylic groups in the polymerization process are discussed. Particle ability to interact with protein was also studied. Monodisperse particles of diameters up to 4 μm had a complex surface layer containing polyvinylpyrrolidone-*graft*-polystyrene copolymers as well as carboxylic groups of the initiator. The effect of this surface structure on the isotherms of adsorption and chemisorption of bovine serum albumin was revealed. Electrophoretic mobility of the particles and their isoelectric point values before and after protein binding depending on pH and ionic strength were determined. These data depend on conditions of particle preparation as well as on protein coating values.

Keywords: Dispersion polymerization of styrene; Polyvinylpyrrolidone-*graft*-polystyrene copolymers; Surface properties of monodisperse carboxylated particles

1. Introduction

Monodisperse polymer particles are widely used in medicine and biotechnology as carriers or sorbents of biologically active compounds, for example, to visualize the immune reactions and, thus, to facilitate the interpretation of their results. The particles for immunological studies must meet some requirements which stimulate the procedures of their preparation with the desired particle size, surface structure, and functionality. The surface of polymer particles can be modified by proteins or other biopolymers via their physical adsorption on the surface of a hydrophobic polymer, usually polystyrene (PS). However, polymer particles with functional surface groups, which provide stronger chemical bonds of immunoreagents, are also rather useful [1]. Carboxylic groups capable of forming amide bonds with the amino groups of bioligands are most frequently used to achieve protein binding. Carboxylated polymer particles of submicron size can be synthesized by emulsifier-free emulsion polymerization of styrene with a carboxyl-containing initiator in aqueous buffers [2,3]. These particles with a rather smooth surface are stabilized by their surface charge controlled by pH and ion strength. Polymer particles of uniform size larger than 1 μm can be prepared by Vanderhoff's successive seed method [4] or by Ugelstad's step-wise swelling method [5,6]. In comparison, dispersion polymerization in polar solvents in the presence of various polymeric stabilizers developed recently is a simple single-step process starting in the monomer solution [7,8]. It is possible to synthesize monodisperse polymer particles with diameters up to 10 μm, using this technique. The main feature of the process is the grafting of growing radicals to polymeric stabilizers. Amphiphilic graft copolymers formed in situ at the beginning of polymerization are more efficient stabilizers than the initial one. They ensure steric stabilization of particle surface after nucleation.

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Therefore, the graft copolymers define the particle size and surface characteristics. Their properties can be varied depending on content of reaction mixture and synthesis conditions, which affect not only particle diameters but also their surface structure.

Here, we report the results of investigation of styrene dispersion polymerization initiated by 4,4′-azo-*bis*-(4-cyanopentanoic acid) (CPA) in ethanol solutions of polyvinylpyrrolidone (PVP). It seems to be a suitable method for preparing monodisperse PS particles of diameters larger than 1 μm with carboxylated and hydrophilized by PVP surface. These particles can be stabilized by ionized carboxylic groups as well as by steric repulsion of PS-grafted PVP chains localized on the particle surface. Moreover, the possibility of quantitative determination of terminal carboxylic groups provides the opportunity to study the effect of synthesis conditions on the structure of polyvinylpyrrolidone-*graft*-polystyrene (PVP-*g*-PS) copolymers. It was also interesting to clarify the features of protein interaction with the particle surface defining the fields of particle application.

2. Experiment

2.1. Materials

Styrene, toluene, and DMF were purified by distillation using standard techniques. Water was distilled twice. Ethanol was distilled and dried from water according to a method described elsewhere [9]. CPA was purified by recrystallization from distilled ethanol. PVP of MW 35000 was purchased from Pharmacon (St Petersburg, Russia). 1-Hydroxybenzotriazole (HOBT), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (CDI), 2-(*N*-morpholino) ethanesulfonic acid (MES), all of commercial grade from 'Sigma', were used without additional purification. Purified BSA was purchased from Institute of especially pure biological products (St Petersburg, Russia).

2.2. Dispersion polymerization

Dispersion polymerization of styrene was carried out in a four-necked glass reactor equipped with a glass paddle-type stirrer, a condenser, a nitrogen inlet, and a temperature controller. Continuous stirring at a rate of 300 rpm was maintained during the process. All latex samples were prepared using 14.3 mM CPA as an initiator (0.2 wt% of the medium). The reaction temperature was maintained at 78 °C. To vary the polarity of the reaction medium, ethanol or ethanol–water and ethanol–toluene mixtures were used. Tables 1 and 2 list compositions of reaction mixtures and characteristics of PS particles obtained. To regulate the particle size and the surface structure, in the case of ethanol–water (93:7 vol%) mixture, the initial concentrations of styrene and PVP in the reaction medium were varied in the ranges of 0.77–1.92 M and 5–30 g/l, respectively. Styrene conversion was established by gas chromatography.

2.3. Washing

The latexes obtained were washed by successive centrifugation followed by redispersion in water (at least three times). Finally, the samples were ultrasonicated and kept in water as 10 wt% suspensions. To analyze the surface layers of PS particles obtained under the conditions of various reagents concentrations, PS microspheres were thoroughly washed with ethanol and 10 mM NaOH in ethanol at 60 °C during 5 h to extract PVP-*g*-PS copolymers. Surface concentration of carboxylic groups before and after each washing step as well as their concentration in washing solutions were determined by conductometric titration [10].

2.4. Latex particle characterization

Latex particle size was measured by transmission electron microscopy (Jeol JEM 100 S microscope, Japan). The polydispersity index (PDI) and the root-mean-square deviation (σ) of all latex samples were calculated on the basis of diameters of more than 400 particles. The molecular weight (MW) was calculated from the data of intrinsic viscosity measurements using Mark-Kuhn-Houwink equation with parameters $K=4.16\times10^{-5}$, $\alpha=0.788$ (toluene) and $K=3.18\times10^{-4}$, $\alpha=0.603$ (DMF) [11]. The electrophoretic mobility of the particles before and after washing as well as after their coating with BSA was studied using standard microelectrophoresis at NaCl concentrations of 1 and 10 mM over a wide pH range 2.0–10.9. Before measurements, the equilibrium between particle surface and NaCl solutions of different pH was achieved for 24 h.

2.5. Characterization of polymer fractions extracted from the particle surface

Polymer fractions removed from the surface were analyzed by two dimensional thin-layer chromatography in comparison with PS of MW 20000 and initial PVP. This analysis proved the absence of homopolymers according to [12]. Isolated PVP-*g*-PS copolymers were analyzed by FT-IR spectroscopy (Bruker-IFS-88 IR-Fourier-spectrophotometer) in comparison with PS, PVP and their mixtures used as reference. Samples were prepared by casting films from various solutions. Analysis of their spectra was based on the characteristic band of C=O deformation oscillations of lactam groups of PVP (1674 cm^{-1}) and of C–H oscillations of PS benzene rings (700 cm^{-1}) [13].

2.6. BSA chemisorption and adsorption on the particle surface

The particles were activated with CDI and HOBT using 10 mM MES buffer (pH 5.5), at 0 °C, 15 min reaction time,

Table 1
Effect of polarity of reaction medium on characteristics of PS particles

Sample No	Medium (vol%)[a]	Styrene (M)	D (μm)	PDI	σ (%)	MW 10^{-4}	
						DMF	Toluene
1	Ethanol–water 93:7	1.44	1.25	1.002	4.4	5.5	7.6
2	Ethanol–water 93:7	1.92	1.88	1.004	6.2	5.7	12.0
3	Ethanol	1.92	2.75	1.019	13.5	5.4	6.9
4	Ethanol–toluene 93:7	1.92	4.84	1.022	15.0	3.2	3.2

[a] [a]Concentrations: 14.3 mM CPA, 20 g/l PVP

according to two-step method described in [14]. CDI and HOBT were taken in equimolar ratio to the surface carboxylic groups. After the activation step centrifugation was applied to remove soluble admixtures, particles were redispersed in MES buffer and BSA solutions (0.05–1.50 g/l) in the same buffer were added. BSA chemisorption on the particle surface was carried out at 20 °C during 24 h, BSA adsorption was carried out under the same conditions but without the preliminary activation step. BSA concentrations before and after sorption were checked by high-performance monolithic chromatography (HPMC) using anionic CIM® DEAE disks (BIA Separations, Ljubljana, Slovenia) [15]. Particles coated with BSA were redispersed for storage in the phosphate-saline buffer (pH 7.4) and were used to study their electrophoretic mobilities.

3. Results and discussion

The decrease in solvent polarity led to increasing particle diameters but, at the same time, the size distribution became broader (Table 1, Fig. 1). A similar dependence has been observed by Thomson and coworkers in the case of initiating with AIBN [8]. The data obtained show that the polymerization mechanism is unchanged in the case of a carboxyl-containing initiator. Weakening of the PVP backbone solvation and increase of PS hydrophobic segments solvation usually result in decreasing stabilizing ability of PVP-*g*-PS copolymers. Moreover, PVP might be less accessible to radical's attack in a weakly polar medium.

It is relevant to review some features of kinetics and the mechanism of dispersion polymerization under investigation. The initial stage is the formation of graft copolymers in solution due to abstraction of hydrogen from PVP chains (Scheme 1). It should be emphasised that Scheme 1 shows grafting PVP by only one PS chain, but the process can be repeated. Scheme 2 shows the structure of obtained PVP-*g*-PS copolymers. At a constant CPA concentration, the number of grafting sites per PVP chain decreases with increasing PVP content in the reaction mixture. The structure of PVP-*g*-PS copolymers formed at low PVP and styrene concentrations might be characterized by many short PS side chains. Their length increases with initial styrene concentration. After nucleation, polymer particles swollen with styrene are stabilized by graft copolymers with terminal carboxylic groups. Grafting can continue at the particle surface. The particle size increases due to absorption of PS chains from solution as well as due to the capture of radicals and polymerization inside the particles. If new particles did not appear in the course of the process, the final particles are rather monodisperse.

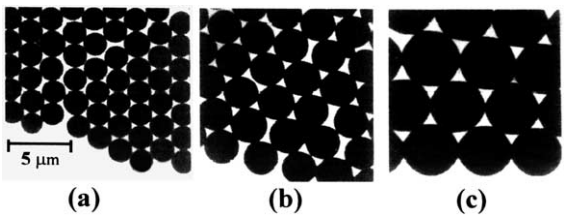


Fig. 1. TEM micrographs of PS particles obtained in (a) ethanol–water (93:7 vol%), (b) ethanol, and (c) ethanol–toluene (93:7 vol%). Concentrations: 1.92 M styrene, 20 g/l PVP, 14.3 mM CPA.

In the case of the ethanol–water mixture, MW, particle diameters and the concentration of surface carboxylic groups were determined at various styrene (Fig. 2) and PVP (Fig. 3) concentrations. The highest surface concentration of carboxylic groups was attained at low styrene concentration (Fig. 2(a)), which is caused by low MW values of PS (Fig. 2(b)). Constant surface concentration of carboxylic groups at higher initial styrene concentration shows that the number of PS side chains with terminal carboxylic groups per PVP macromolecule did not change. At 20 g/l PVP content, the increase in styrene concentration up to 1.15 M (12 wt%) resulted in reduction of particle diameter ensured by increasing stabilizing ability of the copolymers. A similar minimum at 10 wt% methyl methacrylate has been found earlier by Shen and coworkers, who studied dispersion polymerization initiated by AIBN in alcoholic media [16]. At higher styrene content, particle diameter became larger due to reduction of graft copolymers stabilizing ability as a result of better solvation of PS side chains. Such extreme dependence makes it possible to obtain monodisperse particles of the same diameters but with different structures of graft copolymers in their surface

Table 2
Composition of reaction mixtures in ethanol-water medium and characteristics of PS particles

Sample No	Styrene (M)	PVP (g/l)	CPA (wt%) with respect to		D (μm)	Dispersity		$M\times10^{-4}$ (DMF)	[–COOH] (μmol/m^2)		IEP
			Styrene	PVP		PDI	σ (%)		Before extraction	After extraction	
5	1.53	20	2.50	2.00	1.36	1.0011	5.5	3.4	2.36	1.70	2.3
6	1.63	20	2.35	2.00	1.42	1.0018	5.6	4.3	0.99	0.68	2.3
7	1.72	20	2.20	2.00	1.46	1.0038	6.1	6.2	4.26	1.50	2.3
8	1.92	10	2.00	4.00	2.50	1.0013	6.3	3.6	2.72	2.80	2.4
9	1.92	15	2.00	2.70	1.90	1.0013	7.4	3.6	1.39	1.40	2.5
10	1.92	25	2.00	1.60	1.08	1.0470	22	7.8	1.86	1.63	2.2
11	1.92	30	2.00	1.35	1.64	1.0088	9.4	8.2	1.86	1.38	2.2

layer and different surface concentrations of carboxylic groups.

At high styrene concentration 1.92 M (20 wt%) and low PVP content in the reaction mixture, polydisperse particles are formed because of the lack of stabilizing agents (Fig. 3). The increase in PVP content led to smaller monodisperse particles. The surface concentration of terminal carboxylic groups of PS side chains also decreased. This fact confirmed the decrease in the number of grafting sites per PVP chain. The first polymerization stage in solution became longer increasing from 30 to 60 min, as PVP content was changed from 15 to 30 g/l (Fig. 4). This also led to increasing the size of monodisperse particles from 1.64 to 1.90 μm, respectively. In the former case (15 g/l PVP), the increase in polymerization rate indicates that the gel effect took place at conversions over 50%.

MW measured in DMF was in the range of $2–8\times10^4$ increasing with styrene concentration. Under the conditions of a more polar medium (Table 1) and higher styrene and PVP concentrations (Figs. 2(b) and 3(b)), MW values defined in toluene as a precipitant for PVP appeared to be twice higher than the MW values measured in DMF. This fact proved the high probability of grafting PS to PVP. Moreover, the difference between MW values for two solvents indicated that PS side chains became longer. The temperature of initiator addition was also considered as a factor, which allows us to control the structure of graft copolymers and the particle dispersity. Usually CPA was added at 25 °C to the reaction mixtures, which were later heated to 78 °C within 10 min. The diameter of final monodisperse PS particles increased (stabilization became worse) with the temperature of CPA addition: 2.75 μm (25 °C), 3.20 μm (65 °C), 3.80 μm (72 °C). The composition of reaction mixtures is given in Table 1 (sample 3). In the case of CPA addition to the reaction mixture already heated to 78 °C (polymerization temperature), polydisperse latex was formed as a result of weak stabilizing ability of graft copolymers with short PS side chains. Such structure of copolymers is confirmed by the decrease in MW and in the difference between MW values defined in two solvents (Fig. 3 (b)).

FT-IR spectra of PVP-*g*-PS samples removed from the particle surface demonstrated the shift of PVP indicaing band (1674 cm^{-1}) down to 1656 cm^{-1} (Fig. 5). This might be caused by the interaction of PVP and PS at grafting sites. Supposing the shift has not strong effect on the band intensity, mass content of PVP parts in graft copolymers was estimated from the spectra (Table 3). Measured amounts of PVP-*g*-PS copolymers extracted from particle surface and PVP backbone content calculated from spectra decreased with styrene or increased with PVP concentrations in reaction mixtures in parallel (Tables 2 and 3). Thus, synthetic conditions have strong effect not only on the structure of PVP-*g*-PS copolymers, but also on their surface concentration. The average number of PS side chains per PVP chain (n) and the average number of monomer units in

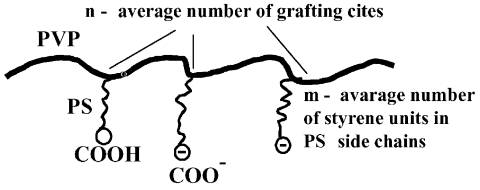
HOOC-I—I-COOH →2 HOOC-I•	Initiator dissociation
HOOC-I• + M → HOOC-I-P_1•	Initiation
HOOC-I-P_n• + M → HOOC-I-P_{n+1}•	Propagation
HOOC-I-P_n• + PVP → PVP• + HOOC-I-P_n-H HOOC-I• + PVP → PVP• + HOOC-I-H	Chain transfer as a result of hydrogen abstraction from PVP
PVP• + M → PVP-M•	Grafting to PVP
PVP-M• + nM → PVP-P_{n+1}•	Propagation of side PS chains
PVP-P_n• + •P_m-I-COOH → PVP-$P_{(n+m)}$-I-COOH	Termination
PVP• + •P_m-I-COOH → PVP-P_m-I-COOH	

COOH-I—I-COOH – 4,4'-azo-bis-(4-cyanopentanoic acid), M – styrene, P – polystyrene, PVP – poly-N-vinylpyrrolidone, PVP-P_n-I-COOH – PVP-*graft*-PS.

Scheme 1. Mechanism of PVP-*graft*-PS formation.

them (*m*) were calculated for three samples obtained under different conditions. The titration data were taken in account considering one terminal carboxylic group per PS side chain (Table 4). The calculated average number of PS side chains per PVP chain varied from 2 to 10. Accordingly, the average amount of monomer units in each PS side chain changed from 96 to 34 and the segments between grafting sites ranged from 100 to 25 monomer units. At high PVP and styrene concentrations in the reaction mixture, only two PS side chains were grafted to one PVP chain, but they contained about 100 monomer units (Table 4, sample 11) and could serve as good anchors. In this case, only 30% of carboxylic groups were removed from the particle surface with graft copolymers, and the mass balance of carboxylic groups was observed. In the case of latex samples 6 and 9 with shorter PS side chains in graft copolymers, 70–100% of surface carboxylic groups were removed from the particles. The surface structure was changed after washing procedure, since terminal carboxylate ions of side chains facilitated extraction. As a result, additional carboxylic groups became accessible for titration. Thus, special control of synthesis conditions allows the preparation of PS particles with various morphology of their surface layer. In particular, the degree of carboxylation, the structure of graft copolymers and the length of hydrophilic tails or loops of PVP can be changed.

Electrophoretic mobility of the particles was investigated



Scheme 2. PVP-*graft*-PS chain.

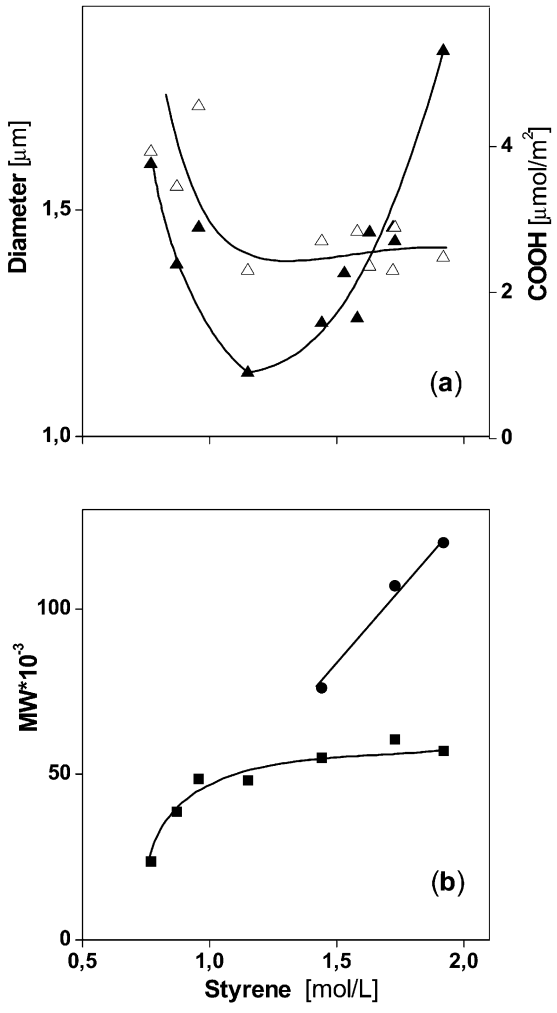


Fig. 2. Effect of styrene concentration (a) on particle diameter (▲), on surface concentration of carboxylic groups (△) and (b) on MW of obtained polymer measured in toluene (●) and in DMF (■). PVP 20 g/l.

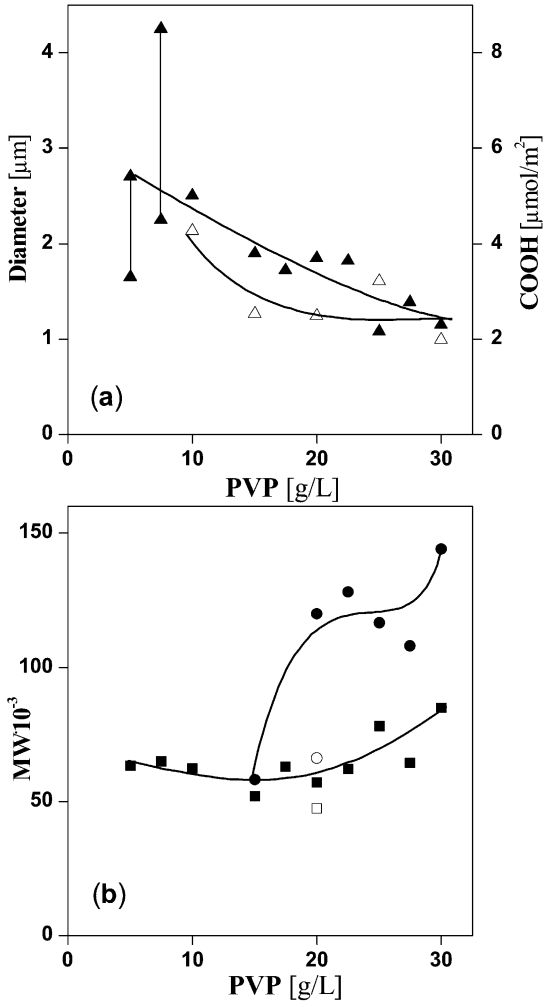


Fig. 3. Effect of PVP concentration (a) on particle diameter (▲), on surface concentration of carboxylic groups (△) and (b) on MW of obtained polymer measured in toluene (●,○) and in DMF (■,□). CPA was added at 25 °C (●,■) and at 78 °C (○,□). Styrene 1.92 M.

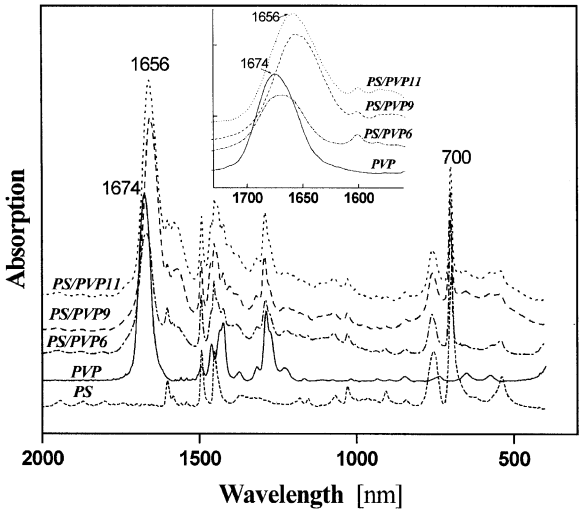


Fig. 5. FT-IR spectra of graft copolymers extracted from PS particles (samples 6, 9, 11) in comparison with spectra of PS and PVP.

for samples before and after washing (Fig. 6). In the case of bare particles, isoelectric points (IEP) were in the range of pH 2.2–2.5 (Table 2), whereas PS particles synthesized with CPA but without polymeric stabilizers in water have no isoelectric point [17]. The ability to inverse the negative surface charge at pH about 2.0 proves the presence of PVP on the surface, since lactam groups of PVP are able to coordinate H^+ at low pH, ensuring the positive particle charge [18]. The lowest electrophoretic mobility values were observed if the particles were washed only with water and their surface was filled with graft copolymers. After their partial extraction, electrophoretic mobilitiy increased, since residual graft copolymers with terminal carboxylic groups of PS side chains could easier stretch toward the slipping plane.

The residual PVP-*g*-PS copolymers with functional groups on the particle surface can serve either as spacers to couple bioligands, or as blockers to protect the surface

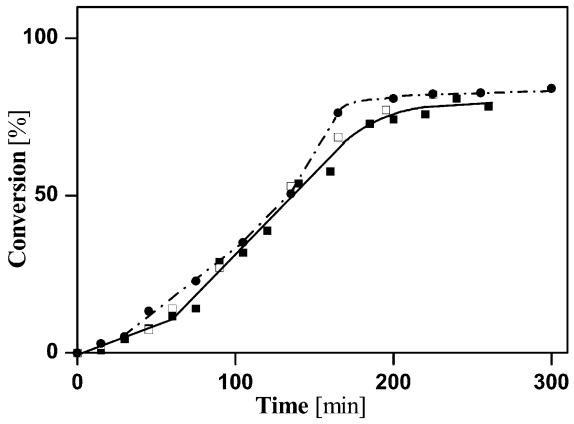


Fig. 4. Change of styrene conversion during dispersion polymerization in the presence of PVP at 15 (●) and 30 (■,□) g/l. Styrene 1.92 M.

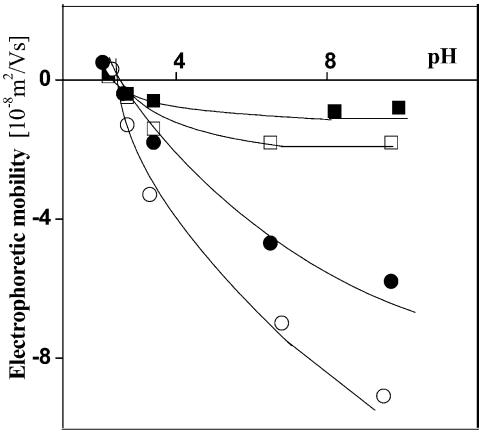


Fig. 6. pH effect on electrophoretic mobility of PS particles (sample 10) before washing (■,□) and after washing (●,○) in NaCl solutions 1 (□,○) and 10 (■,●) mM.

Table 3
FT-IR analysis of graft copolymers obtained by extraction

Sample No	Extracted PVP-*g*-PS		R[a]	PVP backbone	
	wt%	mg/m^2		wt%	mg/m^2
5	1.69	4.0	1.22	53.1	2.12
6	1.47	3.7	0.89	49.5	1.83
7	1.07	2.8	1.19	52.5	1.47
8	0.55	2.4	1.07	49.5	1.19
9	1.01	3.4	1.56	58.9	2.00
11	1.61	4.9	1.64	60.1	2.94

[a] [a]Intensity ratio of PVP to PS indicating bands

from nonspecific hydrophobic interactions. To achieve suitable and accessible localization of bioligands, it is necessary to investigate mixed protein/polymer structures in dependence on electrostatic and steric repulsion in the surface layer. BSA was used as a model protein to investigate the adsorption and covalent binding using water-soluble carbodiimide. Electrokinetic and adsorptive properties were studied for samples 5, 7–9 (Table 2). Fig. 7 shows that the particles obtained are capable of protein binding. The chemisorption and adsorption values attained 3.2 and 2.7 g/m^2, respectively. The same level was also observed in the case of carboxylated PS particles with plain hydrophobic surface [19]. Hence, the PVP backbones of graft copolymers do not interfere with efficient protein binding. At high BSA concentration in solution, chemisorption isotherms are above adsorption ones, except in the case of sample 9. This fact indicates that surface morphology changed with increasing BSA content, when chemisorption achieved 2.5–3.2 mg/m^2. The procedures of activation by CDI and of BSA binding are evidently able to hydrophilize, loosen and extend the particle surface.

Coating with BSA resulted in IEP shifts to the pH range from 3.5 to 5.0. However, the protein's own IEP value (about 4.5–5.0) only was achieved in the cases of sample 5 (at low BSA content) and sample 9. In other cases, lower IEP values proved that surface layers containing BSA have a patchy structure and BSA cannot screen the substrate completely. Thus, the IEP of PS samples coated with BSA depended not only on the BSA content but also on the PVP-*g*-PS copolymer structure. Samples 5 and 7 were obtained at low initial styrene concentrations and the high PVP content. As a result, the structure of the copolymers can be characterized by rather short PS side chains. The decrease in IEP at high BSA content proved that the protein could penetrate under loops of copolymers and settle down on the PS surface (Scheme 3(a)). The decrease in PVP content in the reaction mixture (sample 8) could result in increasing the number of rather long PS side chains in graft copolymers. In the case of sample 9, the number of PS side chains could be smaller but their length could be even longer, as the amount of initiator per PVP chain in the reaction mixture decreased. In cases 8 and 9, the isotherm shapes indicate that, at low BSA content in solution, the copolymers could interfere with BSA chemical binding or with adsorption. However, in the case of sample 9, Fig. 7(d) shows a sufficiently high IEP level even at low BSA content. It was the only studied sample which had practically the same values of IEP and BSA coating both for chemisorption and adsorption. Hence, no noticeable change in surface morphology during interaction with BSA took place. This surface morphology seems to ensure effective BSA binding via hydrophilic spacers deeply anchored into the particle surface by PS side chains(Scheme 3(b)). These data make us hope that protein molecules bound to particle surface will be exposed on the slipping plane and will be accessible for subsequent biological interactions.

4. Conclusion

Dispersion polymerization technique suitable for the preparation of monodisperse PS particles of diameters up to 4 μm as well as methods for controlling latex particle size and surface concentration of carboxylic groups were developed. Polystyrene formed with the carboxyl-containing initiator could graft to hydrophilic

Table 4
Characteristics of PS particles' surface layer

Sample No wt%	Before extraction		After extraction		PVP-*g*-PS obtained by extraction			
	Particles (g)	COOH (10^{-6} mol)	Particles (g)	COOH (10^{-6} mol)	Copolymers (mg)	COOH (10^{-6} mol)	*n*	*m*
6	7.90	31	7.79	21	114	32	9.9	35
9	7.50	31	7.42	32	76	21	6.8	34
11	6.20	38	6.10	28	100	10	2.3	96

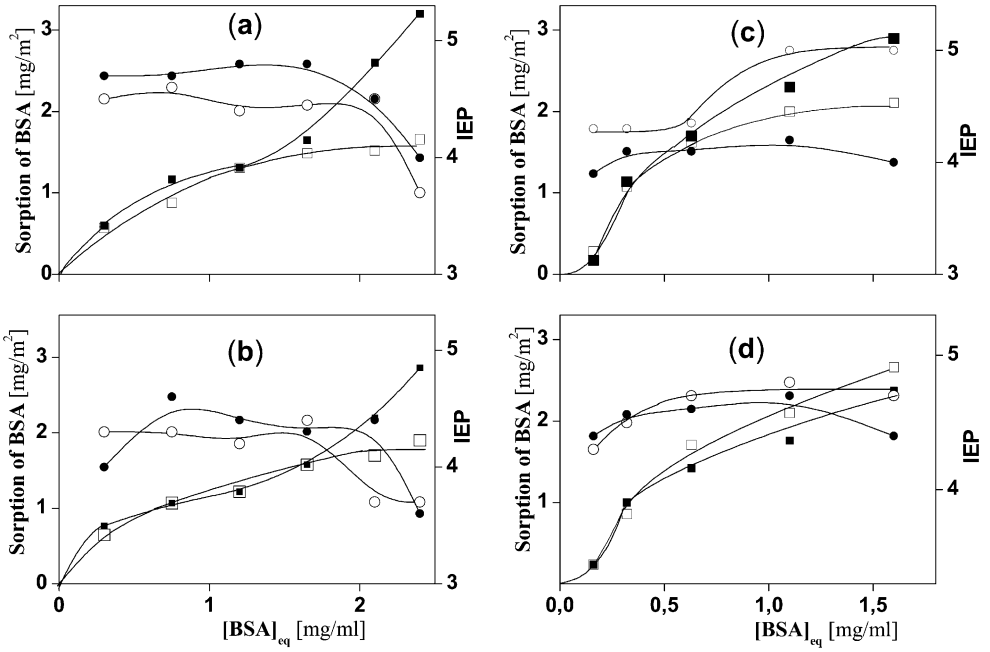
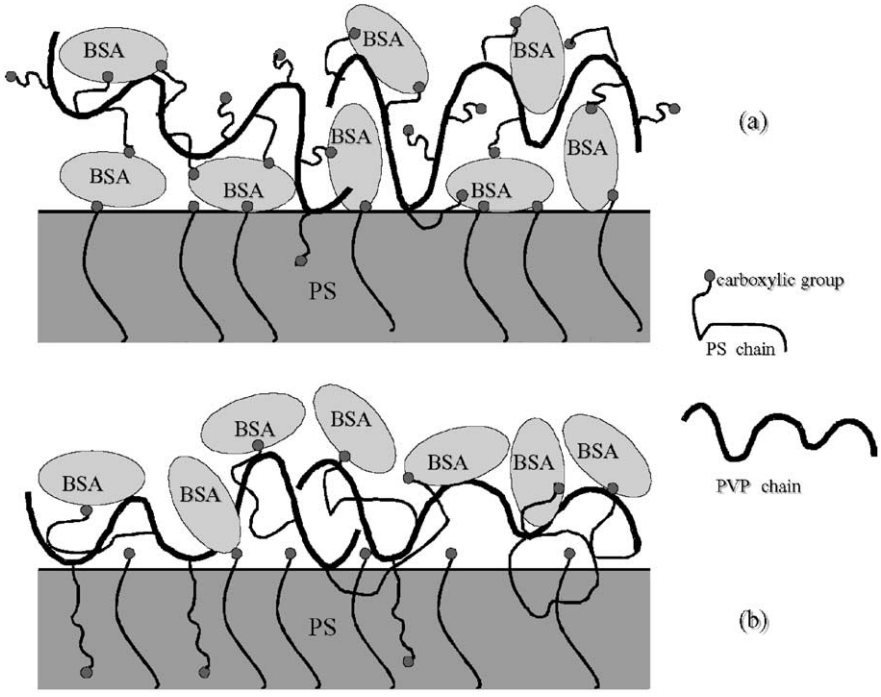


Fig. 7. Adsorption (□) or chemisorption (■) isotherms of BSA and IEP of PS particles after adsorption (○) and chemisorption (●). Samples 5 (a), 7 (b), 8 (c), 9 (d).

PVP used as a polymeric stabilizer in alcoholic media. PS-*g*-PVP copolymers not only stabilize the particles during polymerization but also partially reside on the particle surface even after washing and can participate in interaction with BSA. Their structure studied by FT-IR spectroscopy and conductometric titration was varied with polymerization condition. The particle surface modified by these PS-*g*-PVP copolymers was capable of chemical binding and adsorption of protein at high level surface coverage. Electrophoretic investigation proved that PVP chains and protein molecules bound to the particles influence their surface properties. The results obtained are useful for determing the surface structure of polymer carriers. This can ensure effective



Scheme 3. Structure of washed particle surface after BSA coating: cases of (a) a large number of short PS side chains and (b) a small number of long side PS chains grafted to PVP chain.

proteins binding and localization on the surface being attached via hydrophilic spacers, which retain their biological activity.

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