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Synthesis and characterization of cationic amino functionalized polystyrene latexes

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Abstract: Amino-functionalized latex particles were obtained batchwise by emulsion copolymerization of styrene and vinylbenzylamine hydrochloride (VBAH) in the presence of 2-2′-Azobis(2-amidinopropane) HCl (V50). Size monodispersity of the particles was improved by using divinylbenzene (DVB) as a third monomer at a 2% molar ratio. Surface amino group titration was performed spectrometrically with N-succinimidyl 3-(2 pyridyldithio)propionate (SPDP). The yields of functional monomer incorporation were up to 85%.

Key words: Emulsion polymerization – functionalized particles – amino groups – antibodies – DNA probes – immobilization

Introduction

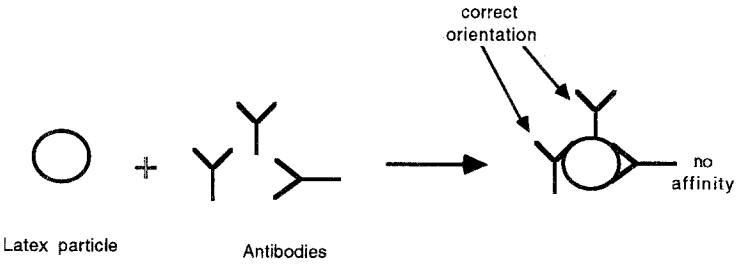
In the field of diagnostics, polystyrene particles are widely used as solid supports for the immobilization of biologically active macromolecules such as antibodies, for example [1].

Passive adsorption has long been used to bind antibodies on polymeric beads. The technology is easy to perform, often gives good results, but desorption of the protein and partial denaturation (loss of specific activity) are the main drawbacks of this process. Therefore, many covalent coupling procedures have been developed (1) using different reactive groups on the proteins and on the particles. The most familiar technique involves the amino groups of the protein to add to activated esters of carboxylic acid groups present on the surface of functionalized latex particles. Nevertheless, amino groups are randomly distributed along an antibody molecule, so we can find antibodies immobilized the wrong way, resulting in a loss of immunological activity.

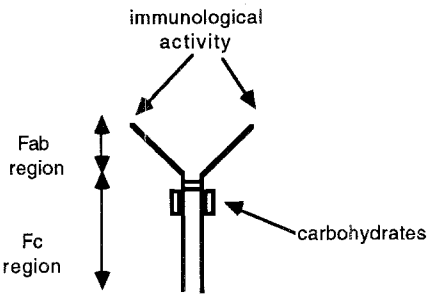
On their Fc regions, antibodies bear carbohydrates which can give rise to aldhehyde groups on oxidation with potassium periodate (2). Therefore, this approach seems appropriate to perform an orientated coupling of immunoglobulins onto amino functionalized particles.

In order to carry out this kind of immobilization, it was necessary to have aminated particles. A preliminary study, based on the use of vinylbenzylamine *1* itself to functionalize seed polystyrene particles was not successful (3), probably due to the reactivity of the hydrogen atoms of the primary amine. In order to improve the functionalization of the particles as well as the stability of the resulting latex it appeared to us that it was necessary to protect the amino group before polymerization. One possible protection was the hydrochloride *2* obtained according to scheme 3.

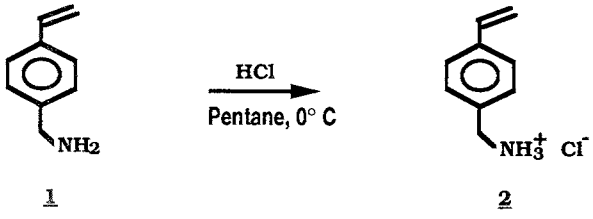
This monomer *2* (called VBAH) is a solid crystalline product, insoluble in styrene. Therefore, we decided to use the same batch polymerization process developed for the synthesis of thioprotected particles with vinylbenzylchloride thiouronium salt (VBIC) (4, 5). In this report the syntheses of cationic latex particles are first described using VBAH as comonomer and with azo-bis(isobutyramidine hydrochloride) as an initiator, then the surface amine groups of the final latexes are characterized using a colorimetric titration.



Scheme 1



Scheme 2



Scheme 3

Experimental part

Material

Unless stated otherwise, reagents and solvents were used as received, water is of milli-Q grade (Millipore SA, France) and is boiled for 1 h under a nitrogen stream before use. Styrene (Janssen Chemica, France) was distilled under reduced pressure, 2-2′-azobis (2 amidino-propane) dihydrochloride (V-50), kindly provided by Wako Chemicals GmbH, Germany, was recrystallized from water-acetone. Divinylbenzene (DVB) (Aldrich) was used as received.

Synthesis of vinylbenzylamine hydrochloride (VBAH)

Freshly distilled vinylbenzylamine, obtained according to (6), was dissolved in pentane and cooled to 0 °C. 1 N HCl solution in pentane (Aldrich), 1.5 equivalents, was slowly added. Precipitation of the insoluble hydrochloride was spontaneous. After warming to room temperature, the white precipitate was washed with ether and used as such (96% yield). ^{1}H NMR (Brucker AC 200, 200 MHz, D_2O, s = singlet, d = doublet, (coupling constant in Hz), reference is D_2O): 7.5 ppm to 7.2 ppm, 4 ^{1}H aromatic protons; 6.7 ppm, 1 ^{1}H d × d (12 Hz × 18 Hz), 5.76 ppm, 1 ^{1}H d (18 Hz), 5.24 ppm, 1 ^{1}H d (12 Hz) ethylenic protons; 4.06 ppm 2 ^{1}H s benzylic protons.

Copolymerizations

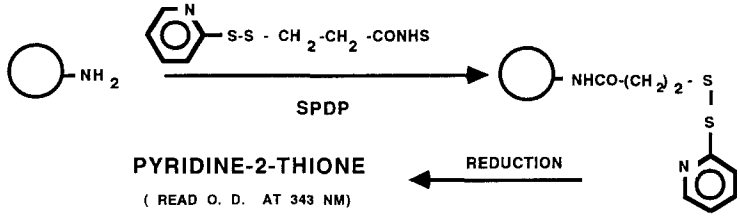
The reactions were performed batchwise in a thermostatted reactor under a nitrogen atmosphere. The required amounts of water, styrene. VBAH, DVB, and magnesium sulfate were brought to the polymerization temperature and left 20 min stirring (350 rpm) at that temperature. Then, an aqueous solution of initiator (V-50) was added. The overall conversions were determined thermogravimetrically and referred to styrene. Particle size and distribution were measured both by quasi-elastic light scattering (QELS, N4MD from Coultronics) and electron transmission microscopy (TEM equipment from Hitachi at the CMABO, Univ. Claude Bernard, Lyon). Photomicrographs were analyzed with a Hewlett Packard 911 A digitalizer. Unless otherwise stated, the diameters reported in the charts are those obtained by TEM.

Latex post-stabilization

Latex post-stabilization was performed as in (4) and (5). Briefly, the required amounts of latex and surface active agents were mixed together and gently rotated end-over-end for 4 h at room temperature before storing at + 4 °C overnight, before use.

Amine titration

Amine titration was performed according to (7) with modifications due to the use of latex particles, see scheme below.



Scheme 4

500 μl of 5% latex suspension were diluted to 1 ml with 0.1 M sodium bicarbonate buffer pH 8.2. 10 μl of a 0.5 M dioxane solution of N-succinimidyl 3-(2 pyridyldithio)propionate, SPDP (from Pierce) were added along with 4-dimethylaminopyridine DMAP (from Aldrich) catalyst. The reaction vials were rotated end-over-end for 100 min at room temperature, then the samples were centrifuged, the supernatants sucked off and kept in separate vials (wash 1). The latexes were resuspended in 1 ml of 50 mM sodium bicarbonate buffer at pH 8,2 and centrifuged again as described. The supernatants were kept aside (wash 2) and the latexes were resuspended as before.

Reduction of the disulfide bond occurred on adding 100 μl of a 50 mM dithiotreitol solution DTT (Aldrich) to the samples and their two washes. After 30 min at room temperature, the samples were centrifuged and the supernatants collected and filtered through a 0.22 μm filter, along with the previous supernatants.

After a 20-fold dilution in 50 mM sodium bicarbonate buffer, the optical density (OD) was read at 343 nm. The amount of available amino groups can be obtained from the coupling yield of SPDP, knowing C_i, the initial concentration in SPDP. The coupling yield Y results from:

$$Y = \frac{A_L}{A_1 + A_2 + A_L}, \quad (1)$$

Where A_L is the OD read at 343 nm for the latex supernatant, and A_1 and A_2 are the OD of washes 1 and 2, respectively.

Therefore, the amount of available amino group is:

[Aminol] (μeq/g latex) = $Y.C_i.P^{-1}$,

where P : the amount of latex, in gram, used in the test;

Ci : initial SPDP concentration (μ moles);
Y : coupling yield (%).

Practically, $C_i = 5$ μmoles and $P = 0.025$ g.

A few remarks are noteworthy concerning the experimental procedure of the colorimetric titration of surface amino groups:

- for each O. D. measurement, the value due to a blank experiment, the latex without any SPDP, is subtracted as to take into account an eventual release in the serum of polystyrene oligomers during centrifugations;
- a reference experiment was run in order to check that SPDP, upon hydrolysis in the medium, would not adsorb non-specifically on the latexes via ionic interactions or hydrogen bonding. In this respect, SPDP was reacted with ethanolamine 1 h before addition of aminated lattices. The results of such experiments show that there was no adsorption;
- preliminary experiments demonstrated that two washes were enough to separate all the unbound SPDP from the particles.

Table 1. Characteristics of latexes obtained at high solid contents.

Run	Latex code	VBNP g/l	[$MgSO_4$, 7H2O] g/l	% Solids	% Conv	Diameter nm	PDI**
1	LV8	0.88	0.0	17.7	97	272	1.003
2	LV6	0.88	0.023	17.5	96	338*	
3	LV5	0.88	0.046	8.7	47	263*	
4	LV4	1.7	0.092	17.2	93	253	1.01

* QELS value.
** value indicated for the major particle population, in the case of a non-monodisperse latex.

Results and discussion

Preparation of the latexes

We were concerned with making latex particles with sizes ranging between 300 and 500 nm suitable for different applications as diagnostics tests.

i) High solid content latexes: The surfactant-free copolymerization process we used at first was derived from that described in preceding papers (5, 6). The recipe was as follows:

V50:	0.14 g (2,6 mmole/1),
styrene:	40 g,
H_2O:	180 g,
[VBAH]:	variable,
θ:	70 °C.

In such a process, a small amount of a highly water soluble monomer, VBAH (ca 0.2% molar referring to styrene), is added to the reaction mixture.

As reported for many cases of emulsifier-free emulsion polymerization in the presence of water-soluble monomers, such polymerization usually proceeds through a complex mechanism (8–10); it generally starts in the water phase involving most of the water soluble monomer and the solubilized styrene molecules. As the radicals grow, they acquire amphiphilic properties depending upon the relative concentrations of both monomers and their reactivity in copolymerization. These oligoradicals play a predominant role in the nucleation step, during which a large number of precursor particles are formed which, associated with a coagulation process, provide mature particles. At this time, the polymerization process mainly takes place in these particles now stabilized by capture of the charged-oligomers; however, aqueous phase termination and styrene depletion in the water phase at high conversion may also cause the formation by hydrosoluble materials and possibly secondary nucleation.

In the present case, it is expected that such a polymerization mechanism also prevails with cationic VBAH since, scarcely soluble in styrene, it exhibits a concentration in the water phase (deduced from the recipes) ranging from 1 to 2.10^{-3} mole/l. In addition, remembering that styrene solubility in water is 2.10^{-4} mole/l, together with probably a stronger reactivity of VBAH in copolymerization (3), oligomers with high VBAH contents should be formed early in the polymerization.

Run 1 (Table 1) provides monodisperse particles as seen from electron microscopy (Fig. 1a). Runs 2 to 4 were devoted to obtaining larger particles by increasing the ionic strength by addition of magnesium sulfate heptahydrate. In run 2, as seen in Fig. 1b, the latex obtained presented small particles together with much larger ones. In run 3, there is too much magnesium sulfate, compared to the amount of VBAH used in the mixture, to let the polymerization proceed smoothly; the yield is low and the resulting latex partially flocculated. So in run 4, the magnesium sulfate concentration and the VBAH concentration were increased. This time, the conversion was correct, better than in run 3, with no flocculated material, proving the stabilizing effect of the cationic monomer, but the latex was still too small in diameter and heterodisperse (indicated in the chart is the size and PDI of the major population of particles).

ii) Low solid content latexes: In order to obtain latexes meeting the size requirements, it was decided to reduce the solid contents of the polymerization medium to ca 5%. Therefore, the recipe was

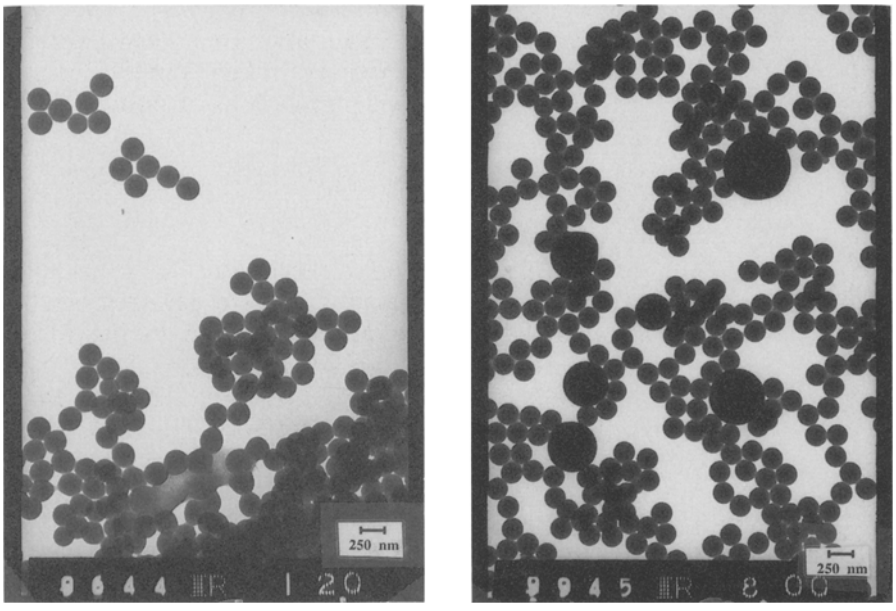


Fig. 1. Electron micrographs of latex particles obtained at high solid contents, from run 1 (1a) and run 2 (1b).

Table 2. Characteristics of latexes obtained at low solid contents.

Run	Latex code	VBNP g/l	[$MgSO_4$, 7H2O] g/l	T °C	% Solids	% Conv	Diameter nm	PDI***
5	LV14	0.79	0.00	70	4.73	89	200*	
6	LV15	0.79	0.105	70	4	74	250*	
7	NC5	0.79	0.21	70	4.4	84	440	1.037
8	LV16	0.79	0.26	70	4.4	84	447*	
9	LV9	0.53	0.21	70	4.1	76	1000*	
10	LV11	0.26	0.21	70	4.4	81	930	1.001
11	LV13-2	0.00	0.21	70	4.1	76	584	1.002
12	NC6-2	0.79	0.21	80	4.7	88	426	1.002
13	NC7-2**	0.79	0.21	80	5.3	97	430	1.004
14	NC8-2	0.53	0.21	80	4.6	85	425	1.001
15	NC 9-2**	0.53	0.21	80	5.1	96	437	1.004

* QELS value
** run with 2% molar DVB
*** value indicated for the major particle population, in the case of a non-monodisperse latex

modified as follows:

H_2O:	180 g
styrene:	10 g,
V50:	0.14 g,
[$MgSO_4$]:	variable,
[VBAH]:	variable,
[DVB]:	variable,
θ:	70 °C (runs 7 → 12),
	80 °C (runs 13 → 18).

On increasing magnesium sulfate concentration (runs 5 to 8, Table 2), larger particles were obtained, but they were rather heterodisperse (except for run 7, but, unfortunately, this experiment was always reproducible).

Effect of the functional monomer concentration on particle size dispersity.

The explanation for high heterodispersity could be that the VBAH concentration was too high:

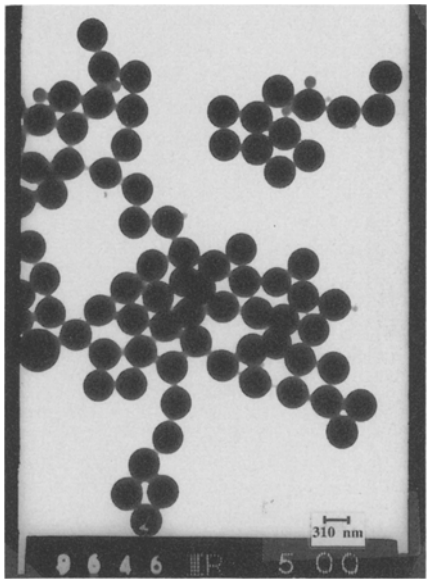


Fig. 2. Electron micrographs of latex particles obtained at low solid contents, from run 10.

– Brouwer et al. (11), with cationic N-trimethyl N-ethylmethacrylate ammonium salts in copolymerization with styrene initiated with V-50, noticed that at higher ionic comonomer content polydispersity increased;
– Kim et al. (12) noticed the same effect in the copolymerization of styrene sodium sulfonate with styrene.

In order to check whether VBAH could be responsible for the lack of monodispersity, experiments 9 and 10 were run with decreasing amounts of functional monomer (respectively two-thirds and one-third of the amount in run 8). The heterodispersity of the particles was reduced but, even with the smallest amount of functional monomer, there were still a few smaller particles beside a majority of bigger monodisperse particles (Fig. 2). In run 11, no functional monomer was added. Close examination of transmission electron micrographs of the final latex shows only a minute amount of much smaller particles, along with larger monodisperse ones (584 nm).

Effect of the magnesium salt on particle size dispersity

Goodwin et al. (13), under conditions similar to those in run 11, but replacing magnesium sulfate by sodium chloride, obtained monodisperse (and monopopulated) particles.

From experiments 5 to 11 it appears that the magnesium sulfate salt and, to a lesser extent VBAH, are both involved in favoring the formation of two different populations of particles.

Effects of the reaction time on particle size dispersity

The presence of a minor population of smaller particles, beside a a majority of larger ones, can be explained by a second nucleation taking place later in the polymerization process. So it was suggested that reducing the polymerization time could be a means of getting monodisperse particles.

According to Fitch et al. (14), the nucleation rate dN_p/dt can be expressed as in (a), where R_i, R_c, and R_f are respectively the rates of initiation, capture and flocculation; and b is an efficiency factor:

$$dN_p/dt = bR_i - R_c - R_f \quad \text{(a)}$$

$$R_i = k_{pw}[M]_w[O]_w \quad \text{(b)}$$

Equation (b) represents the dependence of the initiation rate on the monomer $[M]_w$ and the oligoradical $[O]_w$ concentrations and k_{pw}, the propagation rate constant in water. So, in order to increase the nucleation rate keeping the concentrations unchanged, k_{pw} had to be raised by using either a higher polymerization temperature, or a highly reactive comonomer, DVB for example, (or both).

In this respect, the reaction temperature was raised from 70 °C to 80 °C in runs 12 and 14 and monodisperse particles (diameter 426 nm, $p = 1.002$ in run 12 (Fig. 3a), and 425 nm, $p = 1{,}002$ in run 14) were obtained along with still a few smaller particles.

In a next step to improve the monodispersity by decreasing the reaction time, 2% molar DVB was added to the reaction mixture of runs 13 and 15. At last, particles were monopopulated with a polydispersity index (PDI) of 1,004 as can be seen in Fig. 3b (notice that the surface of the particles obtained with DVB is "rough", whereas without DVB the surface is smooth). Such a result has been already reported in SDS-emulsified emulsion polymerization of styrene (15) as well as in the case of vinylbenzyl chloride under soap-free conditions (16, 17). In contrast, for the emulsion copoylmerization of cationic 3-(methacrylamidinopropyl) trimethyl ammonium chloride

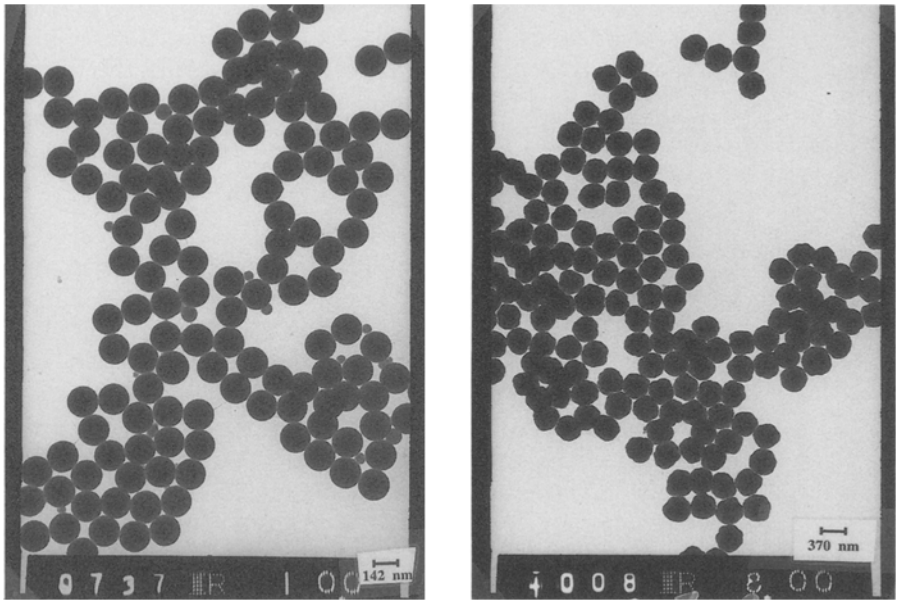


Fig. 3. Electron micrographs of latex particles obtained at low solid contents, at a polymerization temperature of 80°C, without DVB (3a, run 12), in the presence of 2% molar DVB (3b, run 13).

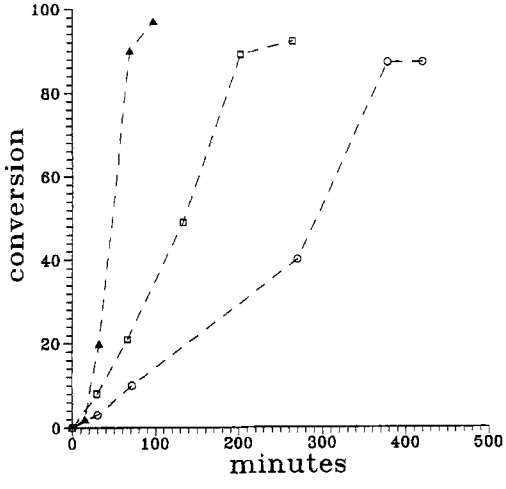


Fig. 4. Comparison of the kinetics of the polymerization reactions of three latexes with identical experimental conditions (except for what is specified in brackets). ○ run 7 (70°C; diam. 440 nm), □ run 12 (80°C; diam. 426 nm), ▲ run 13 (80°C, 2% molar DVB; diam. 430 nm).

with styrene, Streun et al. (18) obtained polydisperse latexes by addition of 1% to 3% of DVB. This striking difference with our result could be explained by the greater reactivity of their functional monomer than that of VBAH.

Surprisingly, temperature and DVB addition had almost no effect on the particle size (Table 2), but only on the polymerization kinetics: Fig. 4 shows the kinetics of runs 7, 12, and 13 performed with the same reactant concentrations but at 70 °C for run 7, 80 °C for run 12, and at 80 °C with 2% molar of DVB for run 13. It took almost 400 min for run 7 to reach 90% conversion, whereas 100 min were required to reach completion in run 13.

From the results above, making monodisperse functionalized particles by copolymerizing styrene and VBAH proved somewhat more tricky than was expected from our previous study with VBIC. It was postulated that a complex nucleation mechanism took place during the copolymerization reaction, with probably the formation of a second crop of particles. This could be partly due to the magnesium salt present in the reaction mixture. This secondary nucleation was suppressed by speeding the polymerization reaction by increasing the temperature and adding a limited amount of DVB. These two parameters effectively shortened the polymerization time without any alteration of the particle size, demonstrating therefore that the determining factor for controlling the particle size is the magnesium salt concentration.

Table 3. Amino group titration using the SPDP method.

Run	Latex code	VBNP g/l	% solid of latex	Diameter nm	μeq/m² experimental	μeq/g experimental	μeq/g theoretical	% yield
1	LV 8	0.88	17.7	272	1.27	28	28	not determined
2	LV 6	0.88	17.5	310	1.45	26	28	for details
4	LV 4	1.7	17.2	253	1.4	33	54.7	see text
11	LV 13-2	0.00	4.1	590	3.15	23	0.00	
13	NC 7-2	0.79	5.3	430	6.47	90	89.6	75
15	NC 9-2	0.53	5.1	435	5.22	72	57.7	85

* concentration during polymerization process

Surface amine group titration

Surface amine group titration was performed on different latexes according to the procedure described in the experimental part. The amount of available amino groups on the particle surface is shown in Table 3.

First, it should be noticed that in run 11, where **no** functional monomer was used, there is still a certain amount of reaction with SPDP, probably due to the initiator, V-50, which on hydrolysis in basic conditions produces primary amide groups (19), and might therefore be susceptible to react (or interact via non covalent bonds) with the activated ester moiety of SPDP. Therefore, to assess the incorporation yield of the functional monomer, it is necessary to take into account the effect of the initiator, as pointed out in run 11. In this respect, for runs 13 and 15, the functional monomer incorporation yield can be calculated as follows:

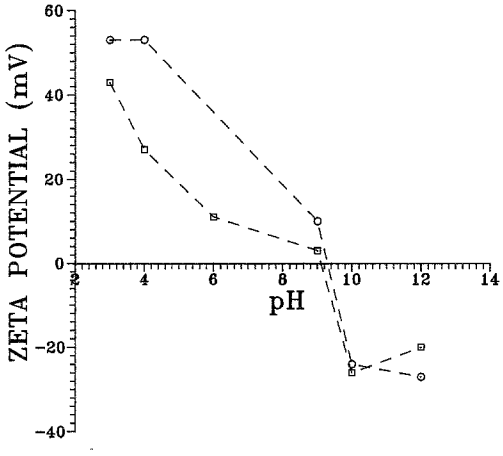


Fig. 5. Zeta-potential of latexes from run 2 (□) and 13 (○) as a function of pH.

$$\text{incorporation yield} = \frac{\text{amount of titrated amine in sample} - \text{amount in run 11}}{\text{theoretical amount from the quantity of functional monomer used in the polymerization process}}$$

Runs 1, 2, and 4 cannot be compared to 11, since they were not produced with the same recipe. With that recipe, we have not been able to polymerize styrene up to completion within 20 h at 70 °C.

The yields indicated in Table IV for runs 13 and 15, are probably underestimated, as it is known that the longer the polymerization reaction, the more initiator is incorporated. It took 7 h for run 11 to reach 76% conversion, whereas 95 min were necessary for runs 13 and 15 to be completed. In addition, these high yields might also be originated by the presence of the crosslinker which could prevent the release of low MW VBAH containing oligomers from the particles, a phenomenon recently mentioned for other cationic latexes (18).

Zeta potential measurements:

Zeta potentials of latexes from runs 2 and 13 were measured vs pH, in 2.10^{-3} M NaCl (Fig. 5) solutions. The positive value of both lattices at low pH value decreases as pH increases, proving

therefore that labile cationic charges are definitely anchored on the particle surface.

Conclusion

Vinylbenzylamine hydrochloride (VBAH) is a cationic monomer which allowed us to complete the synthesis of amino-functionalized latex particles by copolymerization with styrene and using a batch process.

In the course of that study, it was found that by using 2% molar DVB and a polymerization temperature of 80 °C, the reaction time could be reduced with no effect on the particle size, but monodispersity was improved. It will be of interest to extend such a study using a shot growth process with VBAH, a method which should allow to control the surface amine group independently from the particle size; current work in progress will be published later on.

The SPDP titration method and zeta potential measurements showed that positively charged particles were obtained with a fairly high surface incorporation yield of the functional monomer. These particles are currently under investigation for the immobilization of antibodies and DNA fragments.

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