Colloid & Polymer Science Colloid Polym Sci 272:72–81 (1994)

Synthesis and characterization of cationic latex particles bearing sulfhydryl groups and their use in the immobilization of Fab antibody fragments (1)

T. Delair, C. Pichot, and B. Mandrand

Unité Mixte BioMérieux-CNRS, Lyon, France

Abstract: Emulsion copolymerization of styrene and a cationic monomer vinylbenzyl-isothiouronium chloride (VBIC) with initiation by 2,2′ azobis(2-amidinopropane)-dihydrochloride (V-50) gave monodisperse latex particles. After post-stabilization with cationic or non-ionic surfactants, the colloids were diluted in basic buffers with concomitant deprotection of the sulfhydryl groups. Therefore, coupling reactions on the latex beads were possible. Coupling conditions were determined with Ellman's reagent. ^{14}C iodoacetamide and anti C-reactive protein antibody Fab fragments were immobilized and finally, the immunological activity of the sensitized latex was assessed.

Key words: Emulsion polymerization – functionalized particles – sulfhydryl groups – antibody fragments – immobilization

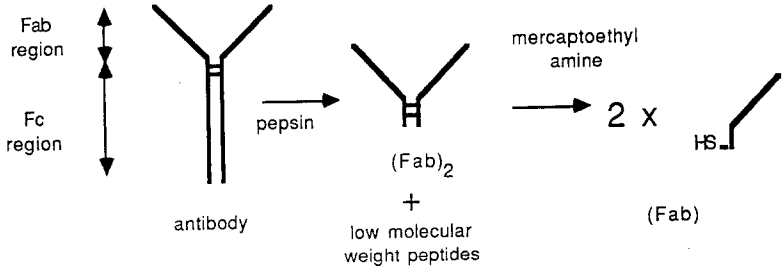
Introduction

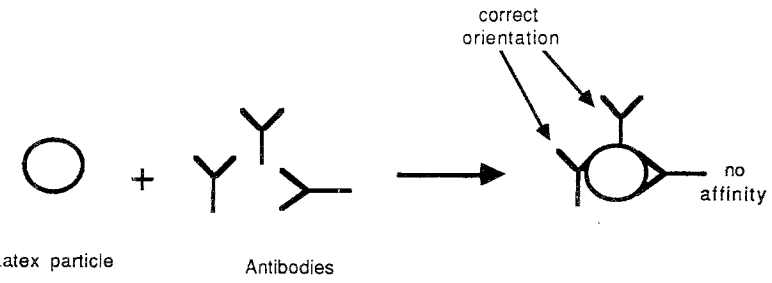
For many years polystyrene particles have been used to immobilize biomolecules, especially in the field of diagnostics [2]. The most widely used particles are of the polystyrene type and those bearing aldehyde, carboxyl and, to a lesser extent, amino groups, in order to allow a covalent binding of the protein onto the latex. In most cases, free amino groups of proteins are involved in the linkage with the particles.

Antibodies are Y-shaped macromolecules, as shown below, where the immunological activity is located at both ends of the Fab parts, the "tail" being called the Fc region.

Amino groups of an antibody (or IgG) are more or less located at random along the macromolecule, depending on the number of lysine residues in the protein. Therefore, coupling involving these amino groups can lead to incorrectly bound antibodies to active sites on the particle, losing immunological activity in the immobilization process. So, it is of great interest to develop an immobilization technique that would ensure a properly orientated coupling, with no loss of biological activity.

From an IgG molecule, pepsin digestion furnishes an $(Fab')_2$ fragments by "eating away" the Fc portion. Those $(Fab')_2$ fragments consist of two Fab fragments held together by disulfide





bonds. Mercaptoethylamine reduction of these disulfide bonds lead to two Fab fragments bearing at one end the immunological activity and, at the other end, at least one free thio group. The sulfhydryl groups at one end of Fab fragments make them very attractive for orientated couplings, via disulfide bond formation, onto polystyrene particles bearing, as well, SH groups.

To our knowledge, there was no commercially available latex functionalized with sulfhydryl groups when we started our work. Yamaguchi et al. [3] reported the emulsion copolymerization of vinylbenzylisothiouronium chloride (VBIC) with styrene to yield latex particles containing thiol groups. When a cationic initiator was used, the basic hydrolysis of the latex to release free sulfhydryl groups, led to flocculation. A stable dispersion was obtained on basic hydrolysis when the initiator was potassium persulfate, the chemically anchored sulfate groups conferring electrostatic stability to the negatively charged particles.

The copolymerization of VBIC induced by potassium persulfate gave, at least in our hands, non reproducible results leading mostly to a partially flocculated material. Therefore, we directed our efforts towards the cationic-initiated copolymerization of vinylbenzylisothiouronium chloride with styrene and subsequent poststabilization of the latex so as to ensure good colloidal stability even under basic pH conditions.

This paper aims at giving results on: i) the study of the synthesis of thio functionalized particles using VBIC and a cationic initiator: 2,2′-azobis(2-amidino-propane) dihydrochloride (V-50), ii) their post-stabilization with various surface active agents, iii) the methods used to assess the binding capacity of our SH bearing latexes and finally their use in the coupling of polyclonal anti C-reactive protein Fab antibody fragments.

Experimental part

Material

Unless stated otherwise, reagents and solvents were used as received. Water was of milli-Q grade (Millipore S.A., France) and was boiled for 1 h under a nitrogen stream before use. Styrene (Janssen chimica, 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 mixture. Vinylbenzylisothiouronium chloride (VBIC) was synthesized according to [4] and recrystallized from a water/ethanol mixture. ^{14}C labelled iodoacetamide was from Amersham (France).

Anti C-reactive-protein polyclonal antibodies (BioMérieux, France) were digested by pepsin at 37 °C to yield $F(ab')_2$ fragments. Reduction of these fragments with mercaptoethylamine afforded crude Fab fragments. Purification was achieved by high performance liquid chromatography (Kontron Instruments).

Copolymerization of vinylbenzylisothiouronium chloride and styrene

The reactions were performed batchwise in a thermostatted reactor under a nitrogen atmosphere. The required amounts (see Tables 1 and 2) of water, styrene, VBIC, and magnesium sulfate heptahydrate were brought to 70 °C and left for 20 min with stirring (350 rpm) at that temperature. Then, a solution of initiator was introduced. The reactions were run until consumption of styrene was complete or for 20 h at 70 °C. The overall conversions were determined thermogravimetrically and referred to styrene. Particle

Table 1. Effect of initiator concentration on the particle size (T.E.M.) and the polymerization yield. All reactions were run with [VBIC] = 2.78 g/l; [$MgSO_4$; 7 H_2O] = 0.22 g/l; [styrene] = 190 g/l. P.D.I. = Polydispersity index

Entry	V50 (mg/ml)	Diameter (nm)	P.D.I.	% solids	% yield
A1	0.78	219	1.012	19	100
A2	0.44	189	1.002	14	77
A3	0.22	340*		2	7

* QELS value

sizes were measured both by quasi-elastic light scattering (QELS) using a N4MD apparatus (from Coultronics) and by electron transmission microscopy (TEM equipment from Hitachi at the CMABO, Univ. Claude Bernard Lyon). Photomicrographs were analyzed with a Hewlett Packard 911 A digitalizer providing the number and weight average diameters ($\overline{Dn}$ and $\overline{Dw}$ respectively) and polydispersity index (PDI).

Electrophoretic mobility measurements

They were performed at 25 °C with a Zetasizer III from Malvern Instruments, France, after diluting the latexes in 2 mM NaCl. The pH of the buffers was adjusted by adding NaOH or HCl solutions. Each point of the curves is the average of at least three measurements. The mobility values were converted into ζ-potential using the Smoluchowski equation ($U_E = \zeta(\varepsilon/\eta)$, where ε and η are respectively the permittivity and the viscosity of the medium.)

Post-stabilization of latexes

To a 10% solids suspension of latex, various amounts of surfactants were added (ranging from 0.5 to 4 mg/ml). The solutions were gently rotated end-over-end for 4 h at room temperature.

The nonionic surfactants were:

- Triton X-405, *t*-Octylphenoxypolyethoxyethanol, (TX 405) as a 70% aqueous solution from Sigma Chemical company (USA),
- Tween 20, polyoxyethylene sorbitan monolaurate, (T 20) from Bio-Rad (USA),
- Nonidet P 40, an ethylphenolpolyethyleneoxide with an average of 9 moles of ethylene oxide per mole of phenol, from BDH Chemicals Ltd (UK).

The cationic surfactant was: Hexadecyltrimethylammoniumbromide (HTAB) from Fluka Chemika AG (Switzerland). Purity was around 98% as indicated by the manufacturer.

Coupling capacity of post-stabilized particles

A) Method using Ellman's reagent

To 4.5 ml of a 50 mM buffer, 0.5 ml of a 10% solids post-stabilized latex suspension and 0.1 ml of a 4 mg/ml solution of 5,5'-dithiobis(2-nitrobenzoic acid), Ellman's reagent [5], were added. To determine the kinetics of the reaction, 0.8 ml aliquots were pipetted off and centrifuged at 16000 g in an Eppendorff 5415 centrifuge. The optical density (O.D.) of the supernatant was measured at 412 nm against a blank being a dilution of 0.1 ml of Ellman's reagent in 5 ml of buffer. The yield of the coupling reaction is the ratio between the O.D. of the supernatant and the O.D. of a mixture of 0.1 ml of mercaptoethanol and 0.1 ml of Ellman's reagent in 4.9 ml of buffer.

B) Method using ^{14}C labelled iodoacetamide

A 10% solids poststabilized latex dispersion was diluted to 1% solids with a buffer containing

Table 2. Effect of ionic strength on the particle size and the polymerization yield. [styrene] = 190 g/l.

Entry	VBIC (mg/ml)	V50 (mg/ml)	$MgSO_4$ (mg/ml)	Diameter (nm)	P.D.I.	% solids	% yield
C0	0	0.78	0	362	1.002	1.8	10
C1	2.78	0.78	0	102	1.005	17	94
C2	2.78	0.78	0.11	201	1.013	18	98
C3	2.78	0.78	0.22	219	1.012	19	100
C4	2.78	0.78	0.33	256	1.009	14	78
C5	2.78	0.78	0.44	PP		PP	
C6	2.78	0.78	0.94	107*		17	99

*QELS value. PP is for polymer paste

the required amount of partially radioactive iodoacetamide. The vials were rotated end-over-end for 5 h at room temperature and the suspensions were centrifuged at 14000 rpm for 10 mn. The supernatant was removed and replaced by an equal volume of water. The resulting dispersion was centrifuged again. This cycle was repeated three times producing three washes and one latex suspension in water. The radioactivity of the washes and of the latex was measured using a Beckmann LS 3801 scintillation counter and Beckmann Ready Safe Scintillation Cocktail. The yield of the coupling reaction for each experiment is the ratio between the radioactivity measured for the latex and the sum of the radioactivity of the latex and of the three washes.

Coupling of anti C-reactive-protein antibody fragment

0.1 ml of a 10% solids suspension of post-stabilized latex was added to a solution of antibody fragments in 50 mM pH 9.2 Tris buffer in order to obtain a 1% solids solution. The vials were rotated end-over-end for 4 h at room temperature. After centrifugation the supernatants were removed and the sensitized latexes were resuspended in 0.15 M NaCl, 10 g/l BSA (Bovine Serum Albumine), 50 mM glycine buffer. The amount of protein remaining in the supernatants were measured using Bradford protein assay [6]. The differences between the initial concentration and that in the supernatants gave the amount of Fab fragments immobilized on the latex particles.

Immunological activity of the sensitized latex

The procedure is that described in [7]. The detection was achieved with a Fab-peroxidase conjugate and orthophenylenediamine (OPD) as a substrate. The detection of the enzymatic reaction was performed by reading the optical density (O.D.) at 492 nm.

Results and discussion

Synthesis of the latex particles

Various polymerization conditions were carried out in order to assess the role on the particle size and on the polymerization yields, of parameters such as initiator and functional monomer concentrations and ionic strength

A) Initiator concentration

Several groups thoroughly studied the properties of various cationic initiators, as V-50 for example, in the emulsion polymerization of styrene [8]. At first preliminary experiments were carried out so as to optimize the initiator concentration to be used in copolymerizations of VBIC with styrene. As seen from Table 1, the effect of initiator concentration is drastic on the overall conversion after 20 h at 70 °C. It appears that using less initiator than in A_1 leads to incomplete polymerization of the co-monomers.

B) Ionic strength and functional monomer concentration

From Table 2, a qualitative insight can be deduced on the role of the functional monomer concentration in the course of the polymerization reaction. In run C_0 where no VBIC is used, a latex whose particle size is 362 nm was produced along with some coagulum; furthermore, the yield was low. C_0 is to be compared with C_1, in which, in the presence of VBIC, small particles were produced together with a higher conversion and without flocculated material.

This drastic effect can be due to the fact that VBIC is readily soluble in water at 70 °C, and it is probably the first to be involved in the polymerization process in the aqueous phase through the initiator decomposition. Then, by incorporating styrene units, surface active oligomers are produced, creating numerous polymerization nuclei and, therefore, via a limited flocculation step, a large amount of small particles is formed. These results are in agreement with those of other groups who worked with ionic comonomers [9]. In our case, the presence of hydrosoluble oligomers was detected by the following experiment: Ellman's sulfhydryl assay was performed on a latex sample from which oligomers were removed by centrifugation. The O.D. values obtained were smaller by a factor of two than those for a non centrigued latex (see later on, Fig. 8).

The effect of ionic strength is shown in run C_1 through C_5. On adding increasing amounts of magnesium sulfate heptahydrate, the particle size increases from 102 nm to 256 nm (C_1 to C_4). Increasing the magnesium sulfate concentration

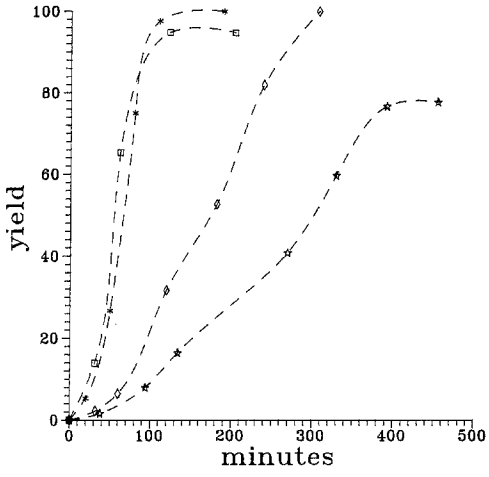


Fig. 1. Conversion plot versus time at different ionic strengths. [$MgSO_4$] = 0 g/l (C_1; □); 0.11 g/l (C_2; ✳); 0.22 g/l (C_3; ◇); 0.33 g/l (C_4; ☆)

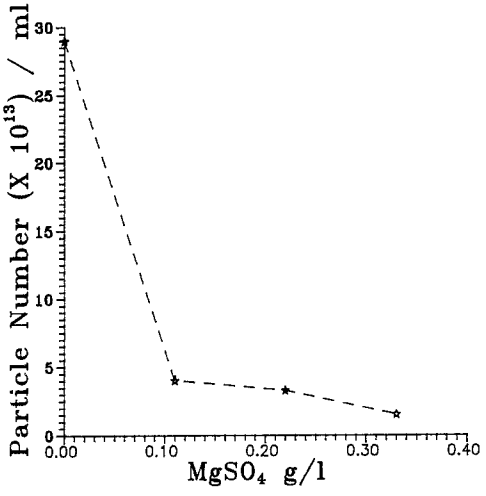


Fig. 2. Number of particles (per cm^3 of latex) after 20 h of polymerization), *Np*, versus magnesium sulphate concentration

higher than 0.33 g/l leads to the production of a polymeric paste (C_5), and the reaction proceeds more slowly. When no magnesium sulfate is added, 94% conversion is reached within 2 h, whereas 20 h are needed to get a 78% conversion with a 0.33 g/l magnesium salt concentration, as seen in Fig. 1. The higher the ionic strength, the smaller the particle number (see Fig. 2) and, therefore, the longer the reaction time.

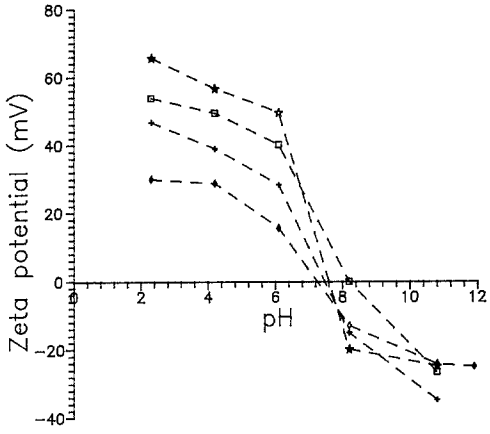


Fig. 3. Zeta potential of latexes versus pH. (C_1, □; C_2, ☆; C_3, +; C_4; ✳)

Surface characterization of functionalized particles

A) Zeta potential measurements

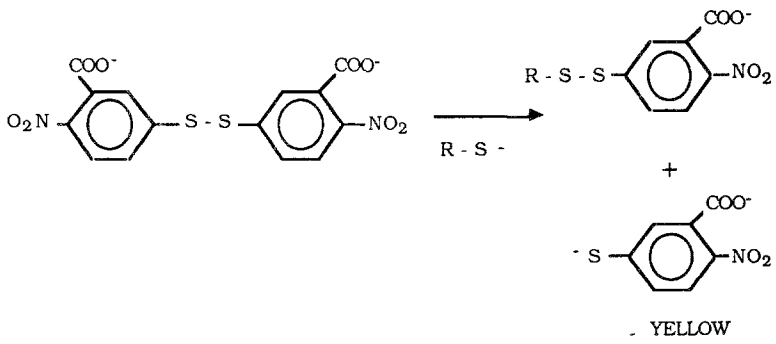
Zeta potential (ζ) measurements were carried out at different pH values on latexes C_1, C_2, C_3 and C_4. Only latex C_4 did not flocculate at pH 11.9.

As illustrated in Fig. 3 which shows the variation of the ζ-potential as a function of pH, the reported curves for all the latexes exhibit an S-shape, with an isoelectric point (i.e.p.) in the pH range of 7.2–8.0 (the uncertainty coming from latex instability in this pH domain). This behavior reflects the contribution of ionic charges coming from the initiator and the monomer; the obtained i.e.p. is near the pH value for which the VBIC units (under cationic form) are transformed into free sulfhydryl groups but also near the i.e.p. (around 7) of amidine-charged polystyrene latexes [10]. Below the i.e.p., an abrupt drop of potential is observed similar to the drop of pH in a strong acid/base titration curve.

In addition, differences in the pseudo-plateau levels according to the sample might reflect variations in the surface charge density of the latexes prepared under increasing ionic strength.

B) Ellman's coupling reaction

This method enables us to study the influence of the nature of the post-stabilization surfactant, the nature of the reaction buffer and the kinetics of the reaction. 5,5′ dithiobis(2-nitrobenzoic acid) (Ellman's reagent) reacts specifically with sulfhydryl groups to yield a disulfide compound and a colored dianion as depicted in the reaction scheme below:



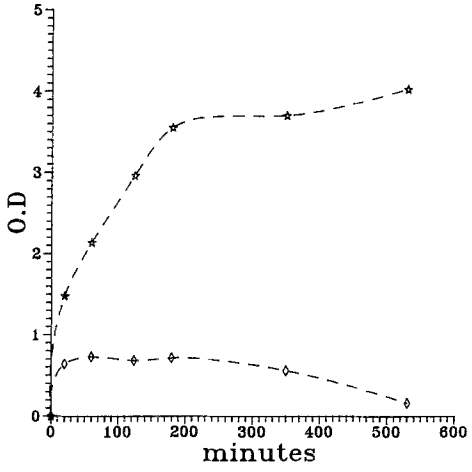


Fig. 4. Effect of the nature of the surfactant on disulfide bond formation on latex C_4 stabilized with triton 405 (TX 405, ☆) or hexadecyltrimethylammonium bromide (HTAB, ◇)

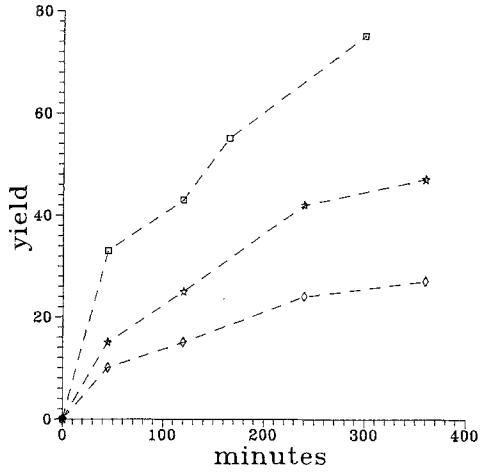


Fig. 5. Effect of the nature of non ionic surfactants (□ Triton; ☆ NP 40; ◇ Tween) on disulfide bond formation on latex C_4

As the reaction proceeds, the yellow color of the supernatant deepens, so the higher the O.D. value at 412 nm, the better the coupling yield.

Experiments were run with latex C_4 diluted to a 10% solids suspension and post-stabilized with 4 mg/ml of hexadecyltrimethylammonium bromide (HTAB) or of Triton X 405 (latexes named C_4 HTAB and C_4 TX respectively).

It appears from Fig. 4, in the case of a stabilization with a cationic surfactant (HTAB), that the signal is lower than with a non ionic stabilizer as TX405. Moreover, a decrease in intensity with time is observed with C_4 HTAB. This is due to electrostatic interactions between the cationic latex and the anionic species released while the coupling takes place, and we end up with a yellow latex and a colorless supernatant.

The nature of the nonionic surfactant is important and Fig. 5 shows that the best signal is obtained with Triton X 405 compared to Tween 20 and NP 40. It is difficult to explain such a result since nothing is known about the absorption behavior of the three different surfactants onto these functionalized PS latexes (molecular surface area, adsorption energy, conformation of the emulsifier at the interface, etc); a kind of systematic study which has not been investigated. Nevertheless, considering that all the surfactants contain PEO chains, it may be postulated that the hydrophobic part of the emulsifier, which is more involved in the adsorption at the particle surface, would confer a more favorable conformation in the case of Triton X 405.

The concentration of the surfactant chosen for post-stabilization does not have much effect on the coupling, as shown in Fig. 6, but is essential to maintain a good colloidal stability. Concentrations inferior to 1.5 mg/ml lead to

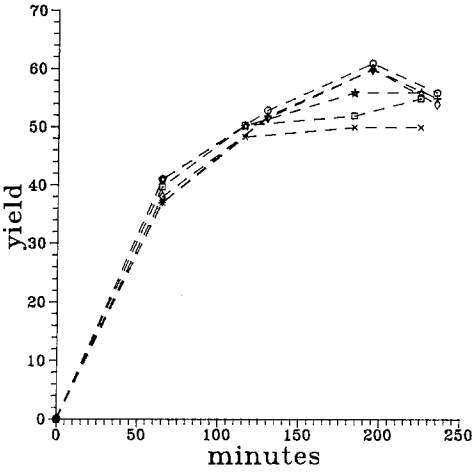


Fig. 6. Effect of the concentration of Triton X 405 on disulfide bond formation on latex C_4 (□; 1.5 mg/ml; ○ 2 mg/ml; ☆ 2.5 mg/ml; + 3 mg/ml; × 3.5 mg/ml; ✳ 4 mg/ml)

Table 3. Ratio (A/PS) of the area (A) occupied by Triton X 405 versus the area developed by one gram of polystyrene at several Triton concentrations

[TX 405] (mg/ml)	Area (A m^2)	A/PS (%)
1	4	18
1.5	6	27
2	9	37
2.5	11	46
3	13	55
4	17	73

autoagglutinated of the latex particles at pH values higher than 7.

Kronberg et al. [11] calculated the specific area occupied by several nonylphenolpolyoxyethylene condensates after adsorption onto latex particles. For a compound bearing 50 ethylene oxide units, the authors calculated an area of 200 A^2/molecule on bare polystyrene latex. Considering:

- this value close enough for Triton X 405, an octylphenol ethylene oxide condensate with an average of 40 ethylene oxide units for one phenol
- that this value of 200 A^2/molecule can be used for functional latexes whose surface characteristics may be very different from those of bare polystyrene

we can calculate the ratio (APS) between the area occupied by the added surfactant (A) (considering that it is mostly adsorbed onto the particles) and the total available area on the latex (PS). The results are summarized in Table 3, taking into account that the total area developed by one gram of latex C_4 is 17 m^2.

At surface coverage percentages inferior to 27% flocculation in the coupling medium occur. Values of surfactant concentrations corresponding to 27% coverage or more have little effect on the reaction itself but they ensure a good colloidal stability (steric-type) with no modification of the initial particle size.

The buffer chosen as a reaction medium is also critical for the reaction to occur, as Fig. 7 points out. No signal is observed in a 50 mM pH 8.8 phosphate buffer, whereas in a 50 mM pH 9.7 carbonate buffer the yield reaches 90%. Reactions in a 50 mM pH 9.2 Tris buffer are slower than in a carbonate buffer but after 400 min reaction time, the overall yields are quite similar.

This method is a means of showing the presence of hydrosoluble oligomers, as indicated earlier. Figure 8 underscores the difference in intensity of a signal due to a non centrifuged and a centrifuged latex sample (latex C_3 centrifuged twice, both sample are post-stabilized with HTAB). The crude signal is twice that of the washed latex, proving

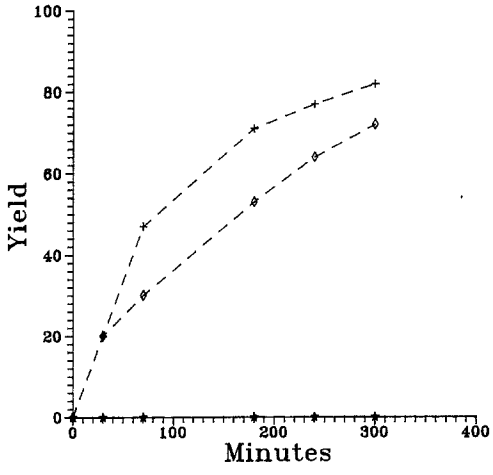


Fig. 7. Effect of the nature of the reaction buffer on disulfide bond formation on latex C_4 (★ Phosphate buffer; ◇ Tris buffer; + Carbonate buffer)

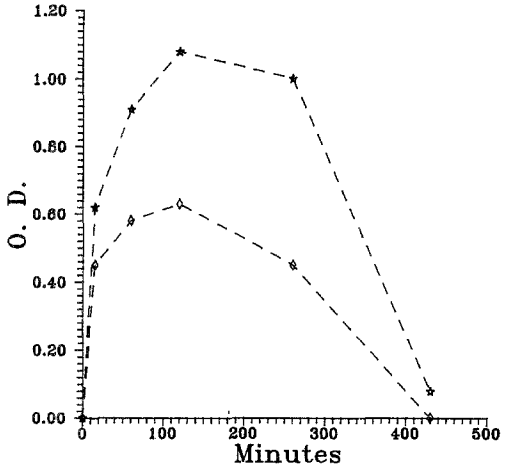


Fig. 8. Effect of washing the latex on disulfide bond formation (latex stabilized with HTAB: ◇ washed latex; ☆ crude latex)

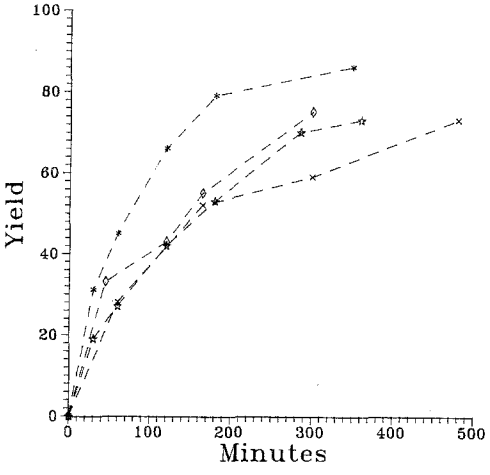


Fig. 9. Chemical stability of surface functional groups of latex C4TX versus time (✳ $t = 1$ day; ◇ $t = 1$ month; ☆ $t = 2$ months; × $t = 14$ months)

that not all the functional monomer was incorporated in the particles during the synthesis. Furthermore, the crude signal value is inferior to the theoretical one, assuming that 100% of the monomer was at the surface of the latex particles, so a part of the functional monomer may also be buried inside the particle.

The chemical stability upon aging of the functional groups on the particle surface was studied; Fig. 9 shows a slight loss of signal of latex C_4TX after 14 months of storing at + 4 °C.

One advantage of this methodology is to allow an insight into the kinetics of the coupling reaction on our sulfhydryl bearing latexes. While the reaction is complete in solution within minutes, it takes at least 4 to 5 h to reach the maximum O.D. value (see Figs. 4 to 6) in the latex. The thio groups at the surface of the particles are sterically more hindered than in solution and so the kinetic constants are smaller [12].

The shortcomings of this method are:

- the observed decrease with time of the yellow color intensity, which makes the use of a reference compulsory (see the experimental section),
- the lack of reproducibility of the signal from one experiment to another, though using a reference helps overcome this problem.

Thus, these factors made quantification of the amount of available surface SH groups difficult. Therefore, there was a need for another, more reliable technique to assess the quantity of thiogroups at the surface of the functionalized particles.

C) Coupling ^{14}C labeled iodoacetamide (IOA)

Initial experiments were run with latex C_4 TX in a 50 mM pH 8.8 phosphate buffer and a 50 mM pH 9.7 carbonate buffer (Fig. 10). The amount of iodoacetamide immobilized reaches a maximum at low initial acetamide concentrations and consequently stays roughly constant with increasing

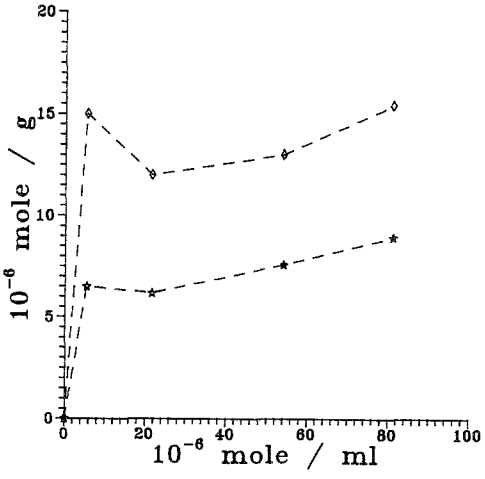


Fig. 10. Effect of the nature of the reaction buffer on immobilization of radiolabelled iodoacetamide (◇ Carbonate buffer, ☆ Phosphate buffer)

Table 4. Amount of available SH group on the particles (in μeq/g of dry polymer) as assessed with 14 C iodoacetamide (IOA) coupling reactions at two different initial IOA concentrations

	54 μmol/ml IOA	5.4 μmol/ml IOA	Mean value	% total VBIC incorporated*
C2TX	28.1 μeq/g	22.8 μeg/g	25.5 μeq/g	47%
C3TX	20.5 μeq/g	17.44 μeq/g	19 μeq/g	35%
C4TX	20.5 μeq/g	19.12 μeq/g	19.8 μeq/g	36%

* Referring to the initial amount of VBIC

concentrations of radiolabeled reagent (Fig. 10). The quantity of immobilized iodoacetamide is lower in the phosphate buffer but slightly increases with increasing concentrations without reaching the plateau value obtained with the carbonate buffer.

With the experimental conditions described above the determination of available surface thiol groups on latex C_2TX, C_3TX and C_4TX was performed, using two different iodoacetamide (IOA) concentrations in the coupling step (5.4 μmol/ml and 54 μmol/ml). The results at 54 μmol/ml (see Table 4) are very close to those obtained at 5.4 μmol/ml, proving that there is not much diffusion of the labelled iodoacetamide inside the particles.

The incorporation yields reported in Table 4 are the ratios of the average value of available surface SH groups per gram of dry polymer to the amount of VBIC introduced per gram of styrene in the syntheses of the particles. The yields range from 35% to 47%, confirming that most of the functional monomer is not at the surface of the particles, but probably in the serum, as shown by the Ellman reactions performed previously, or buried.

D) Antibody fragment immobilization

Antibody fragments were coupled on C_4TX at various concentrations in a 50 mM pH 9.2 tris buffer.

Our experience, so far, in immobilization of antibody Fab fragments, either via covalent bonding or passive adsorption, led to highly autoagglutinated latex. Here, stable colloids were obtained. However, the increase in the particle size, with increasing fragment concentrations, noticed during the coupling step (Fig. 11) is indicative of some partial aggregation.

Immunological activity of bound antibody fragments was measured. The sensitivity is ca

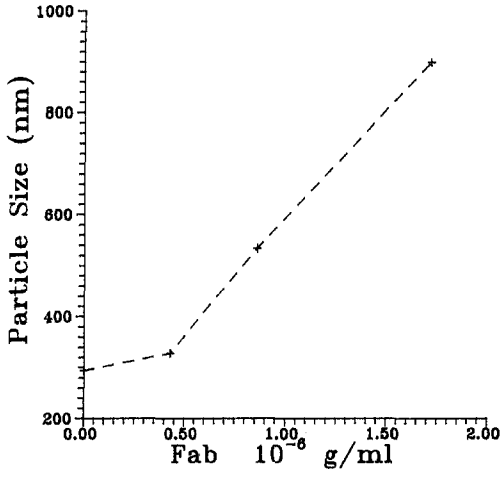


Fig. 11. Particle size versus fragment concentration

500 μg/l, as a concentration of C-reactive protein. In practice there is no need for such a low level since the normal concentration in serum is of 5 mg/l, but these fragments were used as models for further developments.

Conclusions

Emulsifier-free emulsion copolymerization of styrene and VBIC initiated by cationic V-50 made it possible to produce monodisperse functionalized polystyrene latexes with particle sizes ranging from 60 nm to 297 nm. It was shown that ionic strength played a determinant role on the particle size, whereas other factors, such as initiator concentration or VBIC concentration mainly influenced the polymerization yield and the particle stability.

The use of cationic or nonionic surface active agents as post-stabilizers provided stable colloids even in basic media.

With Ellman's reagent, we were able to study the coupling reaction on the thiolated beads and particularly to determine the role of the coupling buffer and to assess the reaction kinetics.

^{14}C iodoacetamide coupling experiments gave access to the amount of available SH groups per gram of latex.

Finally, it was demonstrated that covalent coupling of Fab fragments was available with retention, at least partially, of immunological activity.

Acknowledgements

The authors are thankful to Mrs. M.H. Charles (bioMérieux) for so kindly providing Fab fragments.

References

1. French patent 91.06068 (1991), inv. Th. Delair
2. a) Bangs LB (1985) "Uniform Latex Particles", Technical notes Seradyn; b) Rhône-Poulene Estapor "Diagnostic" booklet, and references therein
3. Yamaguchi K, Watanabe S, Nakahama S (1989) Makromol Chem, Rapid Commun 10:397
4. U.S. patent 3 206 748 (1966), inv. S.J. Nelson Jr.; Chem, Abstr 65:10528c (1966)
5. Ellmann SJ (1959) Arch Biochem Biophys 82:70
6. Bradford M (1976) Anal Biochem 72:248
7. French Patent 90 08825 (1990) inv. D. Darbouret, Charles MH, Theretz A, Mandrand B
8. a) Goodwin JW, Ottewill RH, Pelton R (1979) Colloid and Polymer Sci 257:61; b) Guthrie WH, PhD Dissertation, 1985, Lehigh University.
9. a) Juang MS, Krieger IM (1976) J Polym Sci, Polym Chem Edn 14:2089; b) Liu LJ, Krieger IM (1981) J Polym Sci, Polym Chem Edn 19:3013; c) Brouwer WM, Van Der Vegt M, Van Haeren P (1990) Eur Polym J 26:35; d) Kim JH, Chainey M, El-Aasser M, Vanderhoff JW (1989) J Polym Sci A27:3187; e) Ohtsuka Y, Kawaguchi H, Hagashi S (1981) Polym. J. 22:658; f) Van Streun KH, Belt WJ, Piet P, German AL (1991) Eur Polym J 27:931; g) Twigt F, Piet P, German AL (1991) Eur Polym J 27:939
10. Stoll S, PhD Dissertation, 1992, Université de Strasbourg
11. Kronberg B, Käll L, Stenius P (1981) J Dispersion Science and Technology 2:215
12. Pelton R, "Chemical reactions at the latex-solution interface", in "Scientific Methods for the Study of Polymer Colloids and Their Applications", Candau F, Ottewill RH (eds.) Kluwer Academic Publishers, 1990, p. 493

Received November 5, 1992;
accepted January 28, 1993

Authors' address:

C. Pichot
Unite Mixte BioMérieux CNRS
46 allee d Italie
F 69364 Lyon Cedex
France