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Enzyme immobilization in reactive nanoparticles produced by inverse microemulsion polymerization

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Abstract This paper deals with the immobilization of alkaline phosphatase by physical entrapment within colloidal particles produced by inverse microemulsion polymerization. Functionality has been imparted to the nanoparticle surface by copolymerization of acrylamide (the main monomer), *N*,*N*′-methylene-bis-acrylamide (the cross-linking agent) with either *N*-acryloyl-1,6-diaminohexane (an amine promoter) or acrylic acid (a carboxylic acid promoter). The effect of the functional comonomers on the size and zeta potential of the reactive latexes has been studied. Integrity of the immobilized enzyme has been ascertained from its catalytic activity towards hydrolysis of *p*-nitrophenylphosphate.

Key words Inverse microemulsion polymerization – microemulsion – protein immobilization – latex – surface modification

Introduction

The availability of functional groups on latex particles is highly desirable in many applications, such as paints, adhesives and coatings. Surface properties have indeed a deep effect on colloidal stability and reactivity towards various substrates. This reactivity is of the utmost importance when latexes are used for diagnostic purposes [1] and drug targeting [2].

C. Pichot has recently reviewed the main methods of attaching functional groups onto latex particles [3]. Two general methods are emerging. The first one relies upon the chemical modification of a preformed latex in order to graft the desired functions onto the surface [4]. The most direct and versatile approach however consists in using at least one functional reagent in the latex formulation. Copolymerization is indeed a powerful technique, particularly when a functional comonomer has an intrinsic interfacial activity, so that it spontaneously migrates and accumulates on the particle surface. Hydroxyl [5], carboxyl [6], amine [7], sulfonate [8] and mercapto [9] functional groups have been associated with a latex according to this strategy.

Polystyrene latexes have currently been functionalized for diagnostic applications. A biological probe: an antibody or a nucleotide sequence, is then anchored to the polystyrene particles by physical adsorption or best by covalent bonding [6]. In order to enhance sensitivity and rapidity of non radioactive hybridization assays, we have investigated an original strategy based on the production of a latex containing an enzyme and bearing an oligonucleotide probe on the surface. This enzyme-latex-probe combination is then used in a sandwich-type reaction, as schematized in Fig. 1. The very small size of the particles makes the conditions for the assays reminiscent of solution conditions, so that the reassociation rate is significantly increased. Moreover, the dual duplex is easily recovered by using a second probe bonded to magnetic nanoparticles. Sensitivity of the assay also depends on the immobilized enzyme/probe ratio.

A similar strategy has already been proposed for enzyme immunoassays [10], where both the enzyme and the probe are bonded to the latex surface. The approach

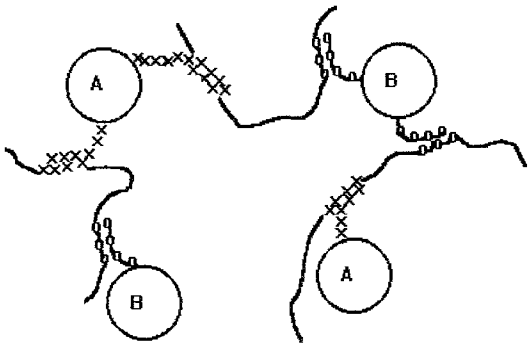


Fig. 1 Hybridization of two nucleic acid segments with probes grafted onto nanoparticles (x) containing an enzyme (A) and nanoparticles (0) containing magnetic particles (B), respectively. Presence of the nucleic acid of interest is thus ascertained by the formation of the A/B duplex, which is easily recovered by a magnet and identified by the enzyme activity

proposed in this work (Fig. 1) is expected to be more efficient, since the enzyme is directly entrapped within the latex which leaves the surface available to the probe and to hybridization. As it will be shown later, this strategy has the additional advantage of increasing the stability of the enzyme immobilized in the latex particles [11]. This feature is particularly favorable to the specificity of the hybridization assay which can indeed be carried out under conditions close to the fusion temperature of the selected hybrid [12].

A preliminary paper has reported on an original way to efficiently immobilize an enzyme: the alkaline phosphatase, into latex particles of a ca. 40 nm diameter, prepared by inverse microemulsion polymerization. This work will focus on the possible functionalization of the latex particles in order to bind the oligonucleotide probe. For this purpose, we have considered the inverse microemulsion copolymerization of acrylamide: the main monomer, *N,N'*-methylene-bis-acrylamide (Bis): the cross-linking agent, and a functional comonomer: either *N*-acryloyl-1,6-diaminohexane (ADH) or acrylic acid (AA). Depending on the functional comonomer, either primary amines or carboxylic acids are expected to be available on the particle surface. The content of the amino or carboxylic acid groups will be measured and the electrokinetic potential of the latex as well. Attention will be paid to the particle size and the properties of the immobilized enzyme.

Experimental part

Chemicals

Acrylamide (AM), *N,N'*-methylene-bis-acrylamide (Bis) and acrylic acid (AA) (Aldrich), ammonium persulfate and *N,N,N',N'*-tetramethylenediamine (TEMED) (Janssen Chimica) were used as received. *N*-acryloyl-1,6-diaminohexane (ADH) was synthesized essentially as reported by Stahl et al. [13]. Sodium bis(2-ethylhexyl) sulfosuccinate (AOT) and polyethylene glycol laurylether (Brij 30) (both from Aldrich) were used as emulsifiers. The enzyme, alkaline phosphatase, was purchased from Boehringer–Mannheim (Germany). Just before use, the protein was dialyzed against water in order to eliminate zinc and magnesium cations added as stabilizers.

Procedure

All the polymerization assays were performed at the same composition of the pseudo-ternary phase diagram, i.e., surfactants: 13.3%; oil: 81%; aqueous solution: 5.7%. In a typical microemulsion polymerization, AOT and Brij 30 were combined in a 34/66 weight ratio and dissolved in hexane. The aqueous phase consisted of ammonium persulfate (117 mM), acrylamide (830 mM), the functional comonomer (ADH or AA) (variable concentration) and *N,N'*-methylene-bis-acrylamide (Bis) (130 mM) in a sodium phosphate buffer (5 mM; pH 7.4). It was dispersed in the oil phase under magnetic stirring. Before polymerization, the aqueous and organic phases were purged with purified nitrogen in order to remove any residual oxygen and the inert atmosphere was maintained during the whole reaction. The microemulsion was cooled down to 0 °C for 5 min, before the polymerization was initiated by the addition of TEMED (33 mM with respect to the oil phase). The reaction was then carried out at 25 °C for 25 min. Latexes were purified by tangential ultrafiltration with a spiral membrane of regenerated cellulose (Amicon) and of a cut-off of 100 000 Da. The polymerization procedure was detailed elsewhere [14].

Latex characterization

The primary amines associated with the latex particles were titrated by visible spectrometry after reaction with trinitrobenzenesulfonic acid (TNBS), as reported by Snijder and Sobocinski [15]. The incorporated AA was titrated by potentiometry. The zeta potential was measured in an aqueous NaCl solution (5 mM, pH 7.0) by a technique based on the Doppler shift of the scattered light (Delsa 440, Coulter Corporation, Hialeah, USA). The particle size distribution was determined by photon correlation spectroscopy (PCS) and by transmission electron microscopy (TEM). The photon correlation spectroscopy equipment was a linear Brookhaven correlator (BI-2030) with 136 data channels and an Exel argon–ion laser

(2 Watt) operating at 488 nm with a 20 mWatt power (vertically polarized light stabilized in the light mode). Time-dependent scattering light fluctuations were measured at 25 °C, with a scattering angle of 90°. Correlation curve data analysis was performed with the Cumulants and Exponential Sampling Softwares supplied by Brookhaven.

The latex particles were observed with a transmission electron microscope (Jeol, JEN SX100) after deposition on a collodion coated copper grid and final coating with carbon at an angle of 45°.

Hydrolysis of *p*-nitrophenylphosphate (10 mM) was used to measure the enzymatic activity of alkaline phosphatase. Concentration of the *p*-nitrophenol was measured by visible spectrophotometry at 405 nm. The enzymatic unit (U) was defined as the number of *p*-nitrophenol micromoles formed per minute at 25 °C and a pH of 9.8 in a $MgCl_2$ (10 mM) containing diethanolamine buffer (1 M).

Results and discussion

Several studies have shown that polymerization in inverse microemulsions is an efficient way to copolymerize acrylamide with polar monomers, such as sodium acrylate [16] methyl methacrylate [17] and also with apolar monomers such as styrene [17]. Copolymerization of acrylamide and sodium acrylate is a representative example of the unique conditions that prevail in inverse microemulsions, since the reactivity ratio of each comonomer is close to one, although these ratios are quite different from each other when copolymerization is carried out in solution or in inverse emulsion [18]. Intrinsic reactivity of the comonomers in inverted micelles is thus modified for reasons not yet clear, possibly due to specific interactions with the microenvironment.

Reaction between the comonomers seems also promoted due to their preferential concentration in the aqueous domains of the microemulsion, limiting so far variations in the comonomer composition at the reaction sites during polymerization.

One of the interests in polymerization in inverse microemulsion and emulsion is the opportunity of immobilizing proteins in latex microgels [19]. Once more, the efficiency of a microemulsion is higher compared to an emulsion, indicating that topology of the polymer latex is significantly different when prepared from a microemulsion rather than from an emulsion [14]. It is thus advantageous to immobilize proteins in microlatex particles, the surface properties of which can be changed and, accordingly, their capability to entrap, transport and release proteins.

Surface characterization

The inverse microemulsion copolymerization of AM with either ADH or AA has been initiated with the redox system, ammonium persulfate and TEMED, in the presence of the cross-linking agent (Bis) and the alkaline phosphatase to be immobilized. Concentration of the functional comonomer has been changed as shown in Table 1. Copolymerization of ADH is quantitative as long as its concentration in the aqueous phase is below 24 mM (Table 1). Above this concentration, not more than 50% of the functional comonomer is incorporated into the latex. When AA is the functional comonomer, there is no clear dependence of the AA conversion on the initial concentration, at least in the investigated range. This comonomer conversion lies between 56 and 69%. This apparently different reactivity of the two functional comonomers is more likely due to a difference in their partition between the phases. We have observed that ADH forms micelles in an aqueous buffer of the same pH and ionic strength as in the aqueous phase of the microemulsion in a concentration range higher than 24 mM. In contrast, micellization of AA does not occur under the same conditions, which indicates that AA is concentrated only in the dispersed aqueous phase, whereas ADH is poorly soluble in this phase.

The electrokinetic potential of the latex particles diluted in water has been measured by Doppler electrophoresis light scattering (Table 2). As a rule, the zeta potential depends not only on the particle nature, but also on the possible adsorption of ions. Since a negative zeta potential is measured for the latex particles whatever the functional comonomer, it must be concluded that the anionic surfactant (AOT) has a decisive effect on that property. Indeed, a positive potential is expected for the particles containing the protonated ADH comonomer at pH 7.0. Although the latex has been thoroughly purified by tangential ultrafiltration and then dialyzed for 2 weeks, the zeta potential is negative. Consistently, latex particles prepared by

Table 1 Conversion of the functional comonomer in relation to the initial concentration

Functional comonomer (initial concentration in aqueous phase)	Comonomer conversion (%)
1. ADH (mM)	
4.8	100 − 5
24	93 ± 4
48	42 ± 6
2. AA (mM)	
50	57 ± 3
250	69 ± 5
500	56 ± 4

Table 2 Electrophoretic mobility and electrokinetic potential for the latex particles, dispersed in NaCl (5 mM) at pH 7. Zeta potential has been calculated in reference to the Smoluchowski approximation (20)

Functional comonomer (initial concentration in aqueous phase)	Electrophoretic mobility $\mu m\ cm\ V^{-1}\ s^{-1}$	Zeta potential (mV)
1. ADH (mM)		
4.8	-1.7 ± 0.2	-21 ± 2
24	-1.5 ± 0.3	-18 ± 3
48	-2.8 ± 0.2	-36 ± 2
2. AA (mM)		
50	-2.5 ± 0.4	-32 ± 4
250	-2.7 ± 0.3	-35 ± 3
500	-2.0 ± 0.2	-26 ± 2

copolymerization of AM and AA have a more negative zeta potential at pH 7.0 than particles in which AA has been replaced by ADH. Thus the charge on the particle surface is at least the combination of two effects: the carboxylate groups of the comonomer and the AOT adsorption. It is also obvious that the surfactant adsorption has a dominant effect on the zeta potential, since no increase in this potential is observed when the AA content in the comonomer mixture is increased. This observation agrees with data reported by Hunter [20] who has highlighted that the electrokinetic potential of a latex could also be influenced by the surfactant adsorption. It should also be kept in mind that the particle charge is sensitive to the nature and concentration of the initiator. Thus, in our case, sulfate anions grafted on the polymer chains might also contribute to the negative charge of the latex.

From the analysis of the electrokinetic potential it is clear that there is a single population of particles, the width of which is essentially independent of the angle at which the Doppler frequency shift is measured, i.e., 7.5, 15, 22.5 and 30 degrees. This observation is consistent with a homogeneous set of latex particles, at least in terms of electrophoretic mobility, and thus in relation to the surfactant adsorption and surface comonomer composition.

Since the zeta potential may not be directly compared to the solid surface charge of the particles, it is of interest to approximate the electrical potential on this surface. The following results have been calculated considering only the charge of the acrylic group. For a 500 mM concentration of AA in the aqueous phase of the inverse microemulsion and a 50% conversion of this comonomer, a surface charge density of 0.08 coulomb/m^2, thus corresponding to 2 nm^2 per charge, can be calculated (see Appendix 1). The calculation has been established for particles of a 40 nm diameter and a density of 1, provided that the AA comonomer is homogeneously distributed within the particle and that the polyacrylic acid is fully ionized. This extreme case is more likely a crude assumption for the situation in the vicinity of the interface where a local acidic microenvironment must prevail in relation to the negative surface potential. Within these limits and considering a surface potential of about 0.4 V (see Appendix 1), a field strength of ca. 4 10^7 V/m would predominate in the region of the particle surface. The possible effect of such a high field strength on the protein is however strongly dependent on the localization and orientation of the protein with respect to the interface. If the protein is completely entrapped within the particle, essentially its activity will be modified by the controlled diffusion of the substrate and reaction products, in particular if they bear ionic groups. Moreover, due to the hydrogel properties on the chemical network (thus an intermediate situation between a liquid and a solid phase), one cannot neglect the fact that the surface potential can partly be dissipated within the particle itself. Would the enzyme be only partly entrapped within the particle, its local conformation in the interfacial region and its activity could be largely influenced by such an electric field strength as membrane proteins are in the biological environment. The migration of the protein towards the interface during polymerization is still reasonable, due to the amphiphilic intrinsic properties of the proteins. In the particular case of alkaline phosphatase, a protein closely associated with the cell membrane [21], it has however been reported that its activity is not significantly affected by a change in the membrane potential [22], the value of which is of ca. 1.2 10^7 V/m under physiological conditions.

As stressed by Hunter [20], if the immobilized protein is partially localized at the particle surface, its presence can provide a more permanent roughness character to the shear-surface layer due to the stability of the protein tertiary structure. Such a crenellated surface would modify the mean local radius of curvature of the particle surface as well as its electrical surface homogeneity, and flexibility. These parameters are known to influence the resulting relaxation and retardation effects associated with the particle motion in the presence of an electric field.

Particle size distribution

Ultra-sound (U.S.) irradiation is needed to disperse the latex particles that have previously been purified and concentrated by lyophilization. After sonication for 3 min, the size distribution of the latex, as analyzed by PCS, does not change anymore. It consists of a major particle population of a 20–40 nm diameter (Fig. 3). A second, although minor, population of particles (ca. 200 nm) is observed, the stability of which suggests that they are non-aggregated species. All the suspensions remain stable at least for 10 days when kept at 4 °C, as proved by PCS analysis.

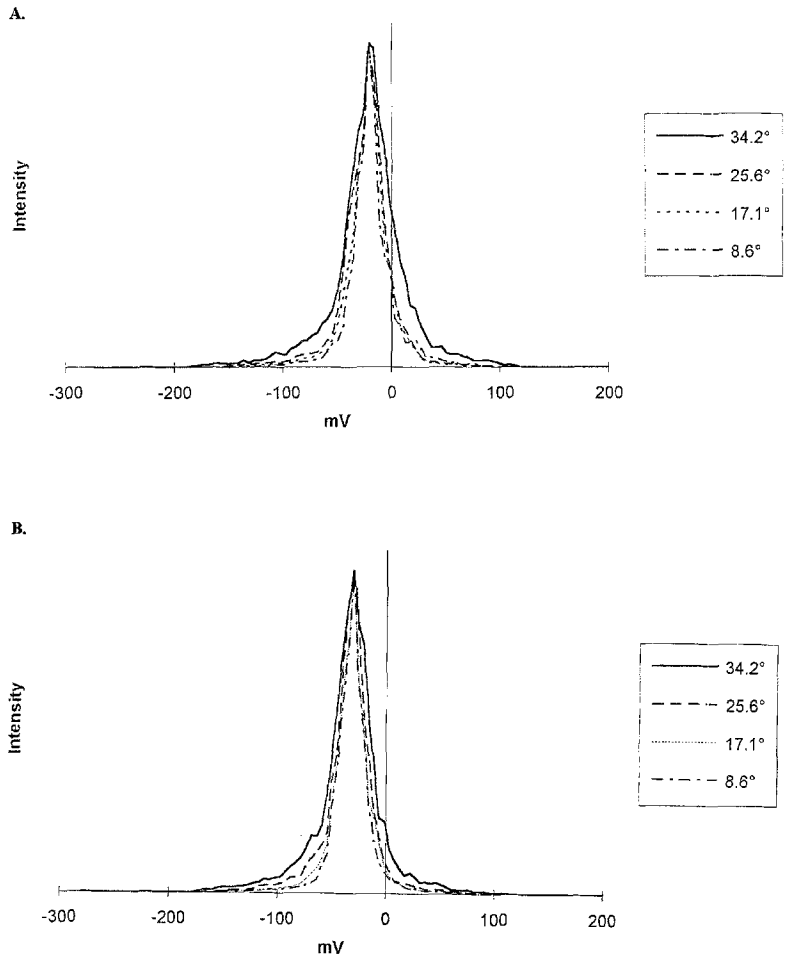


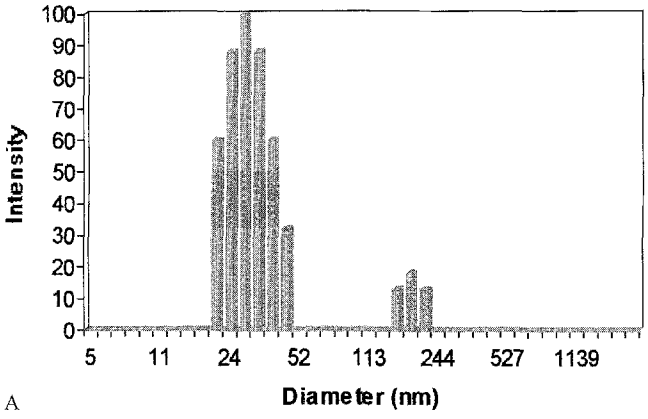
Fig. 2 Zeta potential of latex particles, previously purified by tangential ultrafiltration and dialysis and then irradiated with ultrasound (3 min): A. Aminated latex (4.8 mM: ADH); B. Carboxylated latex (50 mM: AA)

Table 3 Average size of the latex particles (major population) in relation to the initial concentration of ADH and AA in the aqueous phase of the inverse microemulsion

Functional comonomer (mM) in aqueous phase	Intensity average diameter (nm)
ADH	
4.8	28 ± 4
24	40 ± 3
48	50 ± 5
AA	
0.5	40 ± 3
5	30 ± 3
50	38 ± 4
250	33 ± 5
500	39 ± 3
1000	40 ± 6

Table 3 shows that the average size of the latexes containing carboxylic acid groups fluctuates within a range from 30 to 40 nm when the initial AA concentration is regularly increased from 0.5 to 1000 mM in the polymerization medium. The AA comonomer has thus no intrinsic interfacial activity able to control the size of the parent microemulsion. The latex particles with amino groups have a diameter in the same range that, however, seems to increase with the original concentration of ADH in the inverse microemulsion. This apparent control of the particle size by the comonomer concentration can only be accounted for by the amphiphilic structure of ADH and a preferential solubilization in the micellar interface. That ADH behaves as a cosurfactant has already been proposed for explaining the main characteristic features of the pseudo-ternary phase diagrams [14]. Furthermore, the comonomers do not perturb the interface to the point where the interface is no longer stable, as was reported by Antonietti et al. [23].

The final size of the latex particles is 4 to 6 times larger than the original micelles, the diameter of which has been reported elsewhere [24]. This observation is consistent with a continuous particle nucleation mechanism, characterized by a high micelle/radical concentration ratio [25].



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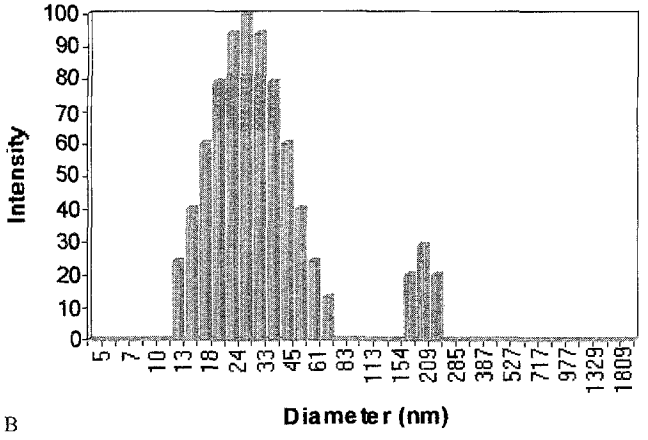

B

Fig. 3 Size distribution of the latex particles, previously purified by tangential ultrafiltration and dialysis and then irradiated with ultrasound (3 min): A. Aminated latex (ADH: 4.8 mM); B. Carboxylated latex (AA: 50 mM)

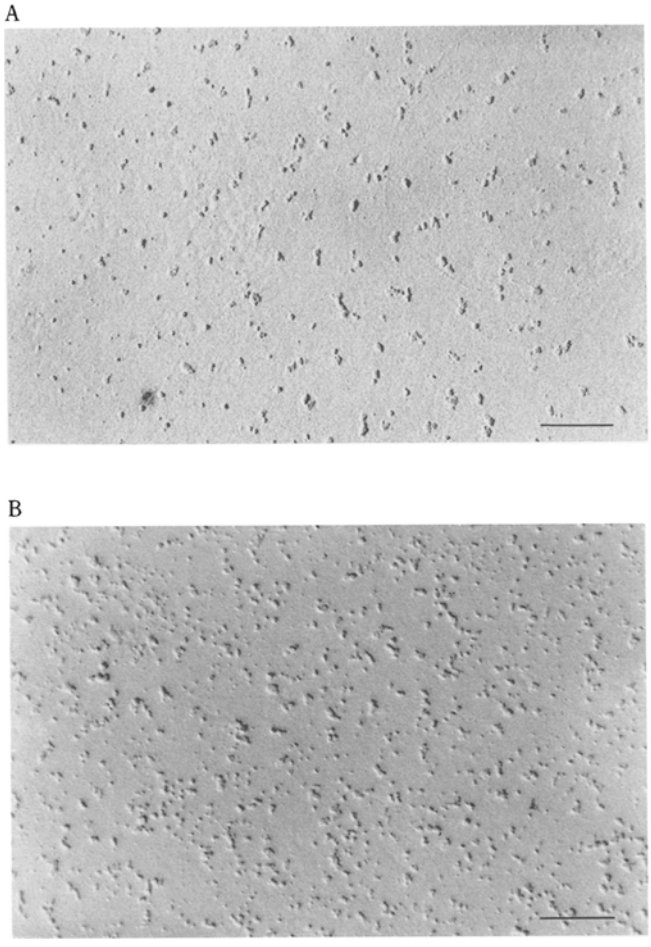

Fig. 4 Transmission electron micrographs of latex particles. Initial concentration of ADH 24 mM (a) and AA 50 mM (b) Bars correspond to 400 nm

PCS data have been confirmed by the direct observation of the latex particles by transmission electron microscopy. Figure 4 shows that most of the particles are spherical and non-aggregated and that their size is smaller than 50 nm whatever the nature of the functional comonomer. Some larger particles or aggregates, of a size exceeding 100 nm, are also observed in a qualitative agreement with PCS.

Enzymatic activity measurements

A previous paper [14] has reported that inverse microemulsion polymerization is a successful technique in order to immobilize an enzyme, such as the alkaline phosphatase, within cross-linked polyacrylamide latex microparticles, while keeping the catalytic activity of the enzyme essentially unmodified. For the sake of comparison, 90% of the alkaline phosphatase is very rapidly released from a gel lattice produced by inverse emulsion polymerization, although 45% of the protein remains immobilized for several weeks within nanoparticles prepared by inverse microemulsion polymerization. This sharp difference in the entrapment efficiency reflects a big difference in the topology of the hydrogel networks prepared from the same initial monomer composition. The chain network formed by inverse microemulsion polymerization thus appears to be more homogeneously cross-linked than the network resulting from the parent emulsion system. Differences in the lattice structure have also been raised by other authors [26].

Since the incorporation of a functional comonomer to latex microparticles is expected to change the local environment, it is worth assessing whether the efficiency of the enzyme immobilization and the catalytic activity of the entrapped enzyme are modified or not. These parameters have been controlled by studying the kinetics of the *p*-nitrophenylphosphate substrate hydrolysis.

Table 4 Yield enzyme immobilization within the latex and corresponding values of the Michaelis constants (K_m) for the hydrolysis of *p*-nitrophenylphosphate

Functional comonomer (mM in the aqueous phase of the microemulsion)	Enzyme activity associated to the latex (% with respect to the initial enzyme amount)	K_m (mM)
ADH		
4.8	43 ± 5	1.14 (± 10%)
24	47 ± 7	1.52 (± 8%)
48	23 ± 4	1.07 (± 12%)
AA		
50	30 ± 3	0.60 (± 5%)
250	28 ± 4	0.73 (± 11%)
500	32 ± 7	0.76 (± 8%)
Free enzyme	–	0.60 (± 13%)

It has previously been shown that kinetics of this reaction catalyzed by the native and the immobilized alkaline phosphatase agrees with the Lineweaver–Burk relationship [14]. Table 4 shows that the Michaelis constant depends on the functional comonomer. When the nanoparticles contain carboxylic acid groups, the enzyme affinity constant (K_M) does not change very much, since K_M increases from 0.6 up to 0.76 mM when the acrylic acid content is increased by a factor of 10. Thus, in spite of a high electric field on the particle surface, the activity of the immobilized enzyme is not significantly perturbed.

This observation has to be compared with data published by Kitano et al. on a significant increase in K_M for the calf intestine alkaline phosphatase chemically immobilized within carboxylated polystyrene latex particles (27). They proposed that the decrease in the enzyme affinity results from electrostatic repulsions between the anionic substrate and the anionic latex. They also assume that the chemical grafting onto the latex results in some conformational restrictions for the enzyme. This conclusion is in qualitative agreement with the result of this study, since the physical immobilization of the enzyme does not perturb the enzyme affinity, although the electrostatic repulsion may not be precluded.

When the latex contains amino groups, there is a twofold increase in K_m compared to the free enzyme, but independently of the initial ADH concentration. The percentage of the enzyme activity within the latex (Table 4) is not modified by the presence of the functional comonomer particularly until an initial ADH concentration of 24 mM.

Conclusions

This paper has shown that inverse microemulsion polymerization is a very suitable method for the production of stable inverse latex particles with primary amine or carboxylic acid groups attached to the surface. These functional groups have been incorporated to the nanoparticles by copolymerization of acrylamide (the main monomer), *N*,*N*′-methylene-bis-acrylamide (the cross-linking agent), and either *N*-acryloyl-1,6-diaminohexane (ADH: precursor of amino groups) or acrylic acid (precursor of carboxylic acid functions). Concentration of the acrylic acid comonomer in the aqueous phase of the microemulsion has no decisive effect on the comonomer conversion (% compared to the initial amount) and the size of the latex microparticles. In contrast, these characteristics features depend on concentration of the amine containing co: monomer in a possible relation to the interfacial behavior of ADH.

The adsorption of the anionic surfactant, AOT, dominates the zeta potential of the latex whatever the functional comonomer (AA or ADH). This potential is negative, although more negative for the acrylic acid containing latex.

In spite of the charges on the latex surface, the catalytic activity of the immobilized enzyme is preserved towards the hydrolysis of the *p*-nitrophenylphosphate.

The next step of this research will focus on the grafting of oligonucleotide probes on the latex surface under conditions that do not perturb the immobilized enzyme.

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Appendix 1

Calculation of the surface charge density for a 40 nm diameter acrylic particle

The weight for one spherical particle is ca $3.3\ 10^{-17}$ g in case of a density of 1. At a 500 mM concentration of AA in the aqueous phase of the inverse microemulsion and a 50% conversion of this comonomer, ca 0.25 g of acrylic acid, i.e., $\sim 2 \cdot 10^{21}$ charges, are incorporated in 1 g of particles (or $3 \cdot 10^{16}$ particles), if the acrylic acid is fully ionized.

On the assumption that the acrylic acid is homogeneously distributed within the particles and that the outer layer of the particle that determines the surface potential is ca. 0.25 nm thick (thus a volume of $\sim 1240\ nm^3$ for a 40 nm particle diameter), it follows that this layer contains ~ 2600 charges for a total surface area of 5000 nm^2. The area per charge is thus 2 nm^2 and conversely the surface charge density is 0.08 coulomb/m^2.

Approximation of the surface potential of the particle and the related electric field strength

Surface potential of the particle (Ψ^0) may be estimated by the next equation (20),

$$\Psi^0 = Q/4\pi\varepsilon_0 Da + Q/4\pi\varepsilon_0 D(a + 1/K)$$

where Q: charge of the interface (coulomb); ε_0: permittivity of vacuum; D: relative permittivity; a: particle radius; $1/K$: Debye–Hückel parameter (estimated to ca 4.3 nm for a 5 mM NaCl solution).

From the total surface charge Q estimated in previous paragraph, Ψ^0 is 0·4 Volt. Therefore, the drop in potential from the interface to the bulk solution (estimated depth of 10 nm) is ca $4 \cdot 10^7$ V/m.

References

1. Tijssen P, Adam A (1991) Curr Opin Immunol 3:233–237
2. Couvreur P (1988) CRC Crit Rev Ther Drug Car Syst 5(1):1–20
3. Pichot C (1990) Makromol Chem, Macromol Symp 327–347
4. Cuatrecasas P (1970) J Biol Chem 245: 3059–3065
5. Tamai H, Hasegawa M, Suzawa T (1989) J Appl Polym Sci 38:403–412
6. Molday RS, Dreyer WJ, Rembaum A, Yen SPS (1975) J Cell Biol 64:75–88
7. Sakota K, Okaya T (1976) J Appl Polym Sci 20:1725–1733
8. Kim JH, Chainey M, El Aasser MS, Vanderhoff JW (1989) J Polym Sci, Polym Chem Ed 27:3187–3199
9. Watanabe S, Nakahama S (1991) Makromol Chem 192:1891–1902
10. Nilsson KGI (1989) J Immunol Methods 122:273–277
11. Daubresse C, Grandfils C, Jérôme R, Teyssié Ph in preparation
12. Hames BD, Higgins SJ (1985) Nucleic Acid Hybridisation: A practical approach. IRL Press England
13. Stahl GL, Walter R, Smith CW (1978) J Org Chem 43(11):2285–2286
14. Daubresse C, Grandfils C, Jérôme R, Teyssié Ph (1994) J Colloid Interface Sci 168:222–229
15. Snyder SL, Sobocinski PZ (1975) Anal Biochem 64:284–288
16. Candau F, Zekhnini Z, Heatley F, Franta E (1986) Colloid Polym Sci 264:676–682
17. Vaskova V, Juranicova V, Barton J (1990) Makromol Chem, Makromol Symp 31:201–212
18. Candau F, Zekhnini Z, Heatley F (1986) Macromolecules 19:1895–1902
19. Schnaar RL, Langer BG, Brandley BK (1985) Anal Biochem 151:268–281
20. Hunter RJ (1981) "Zeta potential in colloid science", Academic Press, London
21. Fosset M, Chappelet-Tordo D, Lazdunski M (1974) Biochem 13: 1783–1795
22. Chappelet-Tordo D, Fosset M, Iwatsubo M, Gache C, Lazdunski M (1974) Biochem 13:1788–1795
23. Antonietti M, Lohmann S, Van Niel C (1992) Macromolecules 25:1139–1143
24. Brajkovic D, "Synthèse de nanoparticules à base de polyacrylamide par polymérisation en microémulsion inverse: application à l'immobilisation d'une enzyme" Graduate thesis, University of Liège, 1993
25. Candau F (1990) Makromol Chem Macromol Symp 31:27–40
26. Antonietti M, Bremser W, Schmidt M (1990) Macromolecules 23:3796–3805
27. Kitano H, Nakamura K, Ise N (1982) J Appl Biochem 4:34–40