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ORIGINAL PAPER

Latex particles by emulsion and dispersion polymerizations: sensitization with specific antigens of leptospirosis and application to immunoagglutination

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Abstract

The controlled synthesis of polymer nanoparticles is of great interest in the production of latexes with well defined characteristics. These products can be applied in biomedicine as carriers of biomolecules (e.g. proteins and enzymes), and in particular as latexes for immunoassays used for example in immunoagglutination test, which allow amplify the antigen–antibody reaction, being simple, quick and inexpensive diagnostic tools. The synthesis of polystyrene (PS) latex particles and of core–shell particles, with controlled size distribution, functional groups and surface charge densities is considered here. PS latexes were synthesized in the 100–1100 nm diameter range, by either emulsion or dispersion polymerization. Such latexes were then used as seeds in emulsion copolymerizations of styrene and a functional monomer (methacrylic acid or glycidyl methacrylate), thus producing particles with carboxyl or epoxy functionalities, respectively. Changes in the polymerization recipes employed under batch operation were analyzed. Latex characterization involved measurements of mean particle diameters, the polydispersity index, functional group densities and zeta potential. Finally, latexes were sensitized (by either physical adsorption or covalent coupling) with specific antigens to obtain latex–protein complexes, and one of them was tested as agglutination assay for detecting leptospirosis disease in bovine samples, as an example of the potential application of the latexes produced.

Keywords Emulsion polymerization · Dispersion polymerization · Immunoagglutination · Leptospirosis

Introduction

Polymeric latex particles for biomedical purposes are of great interest because of their variety of applications (Pichot et al. 1997), which include the production of a latex agglutination test (LAT) for the detection of illnesses like Chagas' disease (Gonzalez et al. 2008b, 2010; García et al. 2013, 2014), cystic echinococcosis (Miranda et al. 2009), bluetongue virus (Yang et al. 2010), neosporosis (Moraveji et al. 2012), toxoplasmosis (Jiang et al. 2008; Peretti et al. 2014, 2016), leptospirosis (Prochetto et al. 2017) and leishmaniasis (García et al. 2017), among others. Leptospirosis is a zoonotic disease caused by the bacterium *Leptospira interrogans* which affects humans and a wide range of animals (Vanasco et al. 2008; Hartskeerl et al. 2011). Diagnosis is performed by either (1) demonstration of the causative agent or its genetic material (direct methods); or (2) serological techniques which detect the presence of specific antibodies (indirect methods). The main indirect detection methods used are the

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enzyme-linked immunoassay (ELISA), and the microscopic agglutination test (MAT).

Various tests have been performed on the basis of particle agglutination principles (Andreotti et al. 2003). In the case of agglutination reactions, it is important that the latexes exhibit a narrow particle size distribution (PSD) for formation of an agglutination network in the presence of the specific antibodies (Ab). Most of the latexes employed for this purpose are based on polystyrene (PS).

Monodisperse PS latexes can be obtained by different synthesis routes, depending on the range of sizes of interest. On the one hand, particles with diameters from 50 to 700 nm are normally obtained by conventional emulsion polymerization (EP). In EP, the monomer (in this case styrene, Sty) is poorly soluble in the aqueous dispersion medium (water). As a result, an emulsion formed by monomer droplets dispersed in water is produced, which is favored by the use of emulsifiers (Em), although PS latexes can also be synthesized without emulsifiers (Li et al. 2001). In the presence of monomer droplets and Em exceeding the critical micellar concentration (CMC), a fraction of the Em (above its water solubility) stabilizes the monomer droplets and the remaining Em forms micelles, which contain monomer dissolved therein. For given agitation conditions, the higher the concentration of Em, the greater the surface that they can stabilize, making it possible to obtain particles of smaller sizes. On the other hand, dispersion polymerization (DP) is an effective method for synthesizing particles of larger diameters, from 500 to 10,000 nm. DP begins in a single homogeneous phase because the monomer, the steric stabilizer and the initiator are all soluble in the dispersion medium. When the initiator is added it decomposes, generating free radicals which lead to the initiation of the polymerization. When the growing polymer chains reach a critical size, they become insoluble and precipitate out of the medium to form polymer particles. The stabilizer molecules play a crucial role to provide colloidal stability in the particle formation, preventing their coagulation (Hong et al. 2007).

In this paper, PS latexes for biomedical applications with narrow PSD and varied particle diameter were obtained by both emulsion and dispersion polymerization. The PS latexes obtained were used as seeds to obtain particles with "core–shell" morphology and different surface functionality (carboxyl or epoxy). Batch operation was considered, in some cases including a shot of initiator or monomers, and different recipes were used for producing latex particles with controlled functionality and charge density. Finally, the PS and carboxylated latexes synthesized here were employed to obtain latex–protein complexes (LPC). To this last effect, total bacterial homogenate (TBH) of *L. interrogans* was utilized and LPC were then tested as LAT reagents for visual detection of leptospirosis in bovine sera.

Experimental

Materials

The following reagents were used in the polymerization reactions: technical grade vacuum distilled styrene (Sty, Pampa Energía S.A., Argentina), methacrylic acid, (MAA, Merk, purity > 99%) and glycidyl methacrylate, (GMA, Aldrich, purity > 97%) monomers. For EP, the emulsifier was sodium dihexylsulfosuccinate (Aerosol MA-80) and the initiator was potassium persulfate ($K_2S_2O_8$, Mallinckrodt, purity > 99%). For DP, polyvinylpyrrolidone (PVP, Sigma-Aldrich, $M_w = 40{,}000$) was employed as steric stabilizer, azobisisobutyronitrile (AIBN, Molekula, purity 99.2%) as initiator, and ethyl alcohol (Anedra)/doubly deionized and distilled water (DDI) as dispersion medium. For both polymerization processes, the buffer was sodium bicarbonate ($NaHCO_3$, Cicarelli). Other reagents used were HCl, NaOH, sodium thiosulfate ($Na_2S_2O_3$), sodium carbonate (Na_2CO_3), and potassium bromide (KBr), all from Cicarelli. For the synthesis of LPC, phosphate buffer (Cicarelli) was used. In the sensitization process by covalent coupling (CC) onto carboxylated latexes, an aqueous solution of *N*,*N*-(3-dimethylamino propyl)-*N*′-ethyl carbodiimide (EDC, Fluka) was prepared shortly before its use. The emulsifier employed for protein desorption was 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (Triton X-100, Sigma). The concentrations of dissolved protein were determined through the copper reduction/bicinchoninic acid (BCA) method. BCA was from Pierce Reagents. DDI water was used throughout the work.

The TBH was obtained from a culture of *L. Interrogans* (Hardjoprajitno strain) as described in Prochetto et al (2017). Bovine serum samples from *L. interrogans*-infected and from non-infected ones, analyzed and classified by the reference technique MAT, were obtained from the "Emilio Coni" Institute (Santa Fe, Argentina).

A solution of polyethylene glycol 8000 (PEG), glycine and bovine serum albumin (BSA), all from Sigma, were used for immunoassay reaction medium formulation.

Synthesis of polystyrene latex particles

A jacketed glass reactor fitted with a stainless steel stirrer and a thermostatic bath was used. Latex particles with carboxyl or epoxy functionalities were synthesized by a two-step emulsion polymerization process. In the first step, PS latexes were synthesized by emulsion or dispersion polymerization. Recipes and conditions of polymerization are shown in Table 1.

Table 1 Recipes and conditions employed in batch emulsion and dispersion polymerizations of styrene; and final conversion and mean particle diameters obtained

Type of polymerization	Emulsion			Dispersion						
Experiment code	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17
Water (g)	556	556	556	–	30.86	40.02	40.14	40.38	40.08	40.12
Ethyl alcohol (g)	–	–	–	125.5	94.04[a]	88.26[b]	89.91[b]	89.80[b]	87.12[b]	86.69[b]
Styrene (g)	234	217	234	18.06	13.60	9.86	9.98	5.16	9.92	9.99
Emulsifier, MA-80 (g)	7.6	11.9	8.8	–	–	–	–	–	–	–
PVP K-30 (g)	–	–	–	3.16	0.91	2.02	2.52	2.50	3.50	3.51
Sty/MA-80 ratio	31.25	18.18	26.32	–	–	–	–	–	–	–
Initiator[e] (g)	0.9	0.9	0.9	0.40	0.22	0.35	0.27	0.27	0.35	0.26
Temperature (°C)	90	90	90	70	70	70	70	70	70	70
Time (h)	12	8	8	8	12	10	10	10	10	10
Conversion, x (%)	98	100	100	67	70	99.9	100	75	95.8	96.6
Particle diameter, D_{DLS} (nm)	300	85	130	–[c]	–[c]	1043	1051	1048	846	445
Polydispersity Index[f]	1.01	1.01	1.01	1.13	1.08	1.09	1.17	1.05	1.22	1.05

$NaHCO_3$ (0.9 g) was employed as buffer in emulsion polymerizations

[a]Isopropyl alcohol/water ratio 3:1

[b]Ethyl alcohol/water ratio 2.2:1

[c]Diameter could not be determined by DLS; diameters obtained by TEM were S11 = 2.90 μm and S12 = 1.74 μm

[e]$K_2S_2O_4$ for emulsion polymerization or AIBN for dispersion polymerization

[f]Obtained after counting 100–500 particles as the quotient between the weight average and the number average particle diameters (Eq. 3)

On the one hand, 3 PS latexes (S8, S9 and S10) were synthesized by emulsion polymerization in the presence of different concentrations of emulsifier (MA-80), with the aim of obtaining latexes with different particle sizes and PSD with low polydispersity index. First, the reactor was charged with a solution containing the buffer $NaHCO_3$, MA-80, the monomer and DDI water. Temperature of the water bath was raised up to approximately 91 °C and the system was stirred under a stream of N_2. After reaching the desired temperature of 90° in the reaction medium, the initiator was added.

According to the classical theory established by Smith and Ewart (1948), the dependence of the final particle number N_p, with the initiator concentration [*I*], and the emulsifier concentration [*Em*] is given by:

$$N_p = k([I])^{0.4}([Em])^{0.6}, \quad (1)$$

where k is a coefficient and N_p can be calculated from Eq. (2) on the basis of the final particle diameter D_{DLS} (in cm), in this case measured by Dynamic Light Scattering (DLS, Brookhaven Instruments Inc.), and assuming a monodisperse PSD:

$$N_p = \frac{6m_{pol}}{\pi \rho_{pol} D_{DLS}^3}, \quad (2)$$

where m_{pol} is the mass of polymer in the final latex (in *g*) and ρ_{pol} is the density of PS (1.04 g/cm^3).

On the other hand, 7 PS latexes (from S11 to S17) were synthesized by dispersion polymerization. For this, the reactor was loaded with the steric stabilizer PVP dissolved in the dispersion medium and Sty monomer, thus forming a single homogeneous phase. The dispersion medium was pure alcohol (S11) or a mixture of alcohol/water (S12–S17), employing isopropyl alcohol for experiment S12 and ethyl alcohol for the other reactions. The temperature was fixed at 70 °C and mechanical agitation with N_2 bubbling started. After 30 min, the initiator AIBN dissolved in a fraction of the dispersion medium was added, and the reaction temperature was maintained at 70 °C. The dispersion medium, PVP, AIBN and monomer concentrations were modified with the aim of obtaining stable latexes of particle diameters between 500 and 1000 nm (Zhou et al. 2013).

Synthesis of functionalized latex particles

Six core–shell latexes with carboxyl functionality were obtained from the PS seeds S8, S10 and S13: S8C1, S8C2, S10C1, S10C2, S13C1 and S13C2, respectively. Also, two latexes with epoxy functionality were synthesized, using the PS latex S8 as seed: S8E1 and S8E2.

The carboxyl latexes were synthesized by emulsion copolymerization of Sty and MAA, following the reaction strategy reported by Gonzalez et al. (2008a). First, PS seeds,

monomers and water were initially charged into the reactor, and the system was kept under stirring at room temperature for 2 h to produce the swelling of particles, and under a stream of N_2 to reduce the amount of O_2. The monomer ratio MAA/Sty was varied to obtain different carboxyl group densities (δ_{COOH}). In half of the experiments (S8C2, S13C1 and S13C2) additions were considered, including a second load of initiator solution after 4 h of reaction to increase the final conversion. Moreover, for experiments S13C1 and S13C2, the following two ingredients were added in the initial load: MA-80 emulsifier and $NaHCO_3$, in order to provide better stability of the particles under an alkaline pH (the MA-80 concentration employed was below the CMC in both cases to avoid the presence of micelles and the production of new particles by secondary nucleation).

The epoxy latexes were synthesized by emulsifier-free emulsion copolymerization of Sty and GMA, where the monomer ratio was varied to control the epoxy group density (δ_{C2H3O}). To increase the final conversion, a second load of initiator was injected after 4 h of reaction. In one case (S8E2), a second load of monomer solution was also injected after 3 h of reaction to increase δ_{C2H3O}.

Recipes and synthesis conditions for the emulsion copolymerizations of Sty/MAA and Sty/GMA are given in Table 2.

Characterization of polymer latexes

For all latexes synthesized in this work, the monomer conversion (x) was first determined by gravimetry as the ratio of the produced polymer to the total fed monomers of samples collected along the polymerizations. Then, the unreacted comonomers and $K_2S_2O_8$ were eliminated by serum replacement to purify the polymer particles. The average particle diameter D_{DLS} was estimated by DLS at a detection angle of 90° through the quadratic cumulants method. The polydispersity index (PI) was calculated for the final latexes from the number PSD determined by Scanning Electron Microscopy (SEM, JEOL-JSM 35C) or by Transmission Electron Microscopy (TEM, JEOL-100 CX II) on representative samples as in Saenz and Asua (1995):

$$\mathrm{PI} = \frac{D_w}{D_n} = \frac{\frac{\sum_{i=1}^{n} N_i D_i^4}{\sum_{i=1}^{n} N_i D_i^3}}{\frac{\sum_{i=1}^{n} N_i D_i}{\sum_{i=1}^{n} N_i}}, \tag{3}$$

where D_w and D_n are the weight and number mean diameters, respectively; N_i and D_i are the total number of particles and diameter of particles of type i, obtained from the PSD estimated by electron microscopy. For the preparation of SEM samples, a drop of diluted latex (about 0.01% w/w) was dried on a glass holder, and an evaporator was used to cover particles with a thin film of gold. For TEM samples, copper grids were used, which were conditioned by applying a Formvar film thereon. Then, a drop of diluted latex (about 0.01% w/w) containing 0.01% of phosphotungstic acid was placed on the grid and allowed to dry at room temperature.

For functionalized particles, the carboxyl group density (δ_{COOH}) was measured by conductometric titration, employing an automatic titrator (KEM, model At-510). For the conductivity measurements, final samples were diluted with

Table 2 Recipes and reaction times employed in the seeded emulsion polymerizations of styrene/methacrylic acid and styrene/glycidyl methacrylate at 70 °C; and main characteristics of the final latexes

Experiment	S8C1	S8C2	S10C1	S10C2	S13C1[a]	S13C2[a]	S8E1	S8E2
Water (g)	461.42	462.73	413.45	414.67	112.03	109.72	94.99	88.54
Seed (g_{pol})	6.49	6.31	5.83	4.43	1.40	1.39	1.50	1.51
Styrene (g)	3.49	4.04	6.72	2.54	0.34	0.28	0.97	1.78[c]
Methacrylic acid, MAA (g)	0.47	0.99	1.02	0.19	0.09	0.13	–	–
Glycidyl methacrylate, GMA (g)	–	–	–	–	–	–	0.42	2.04[c]
MAA/Sty or GMA/Sty ratio	0.13	0.25	0.15	0.08	0.24	0.46	0.43	1.14
Initiator, $K_2S_2O_4$ (g)	1.05	2.10[b]	1.05	1.05	0.52[b]	0.47[b]	0.74[d]	0.56[d]
Time (h)	12	12	12	12	6	6	8	8
Conversion. x (%)	75.6	86.2	49.5	56.4	98.8	93.8	69.3	80.0
Particle diameter. D_{DLS} (nm)	340	354	193	180	1133	1194	363	520
Polydispersity Index[e]	1.021	1.027	1.017	1.029	1.067	1.051	1.016	1.069
δ_{COOH} or δ_{C2H3O} ($\times 10^{-7}$ mEq/cm^2)	5.7	13.0	14.0	3.5	5.1	9.5	0.9	1.7
ζ (-mV)	62.9	52.0	56.1	62.0	39.3	25.9	48.8	43.9

[a]Other reagents were: MA-80: 0.57 g, $NaHCO_3$: 0.27 g

[b]Half was charged at t = 0 h and the rest at t = 4 h

[c]0.90 was charged at t = 0 h and the rest at t = 3 h

[d]0.37 was charged at t = 0 h and the rest at t = 4 h

[e]Obtained after counting 100–500 particles as the quotient between the weight average and the number average particle diameters (Eq. 3)

DDI water under magnetic agitation and an HCl solution was added to produce the complete protonation of the accessible acidic groups (corresponding to the sulfate groups from the initiator and the carboxyl groups from the MAA units). The titrating agent was a NaOH solution. The epoxy group density (δ_{C2H3O}) was determined by potentiometric titration (Sundberg and Porath 1974; Chen and Lee 1999; Hou et al. 2007). This assay is based on a redox reaction between the epoxy groups and sodium thiosulfate ($Na_2S_2O_3$), leading to the production of OH^- proportional to the amount of oxirane rings. The titrating agent was an HCl solution.

The zeta potential (ζ) was determined at pH 6 by measuring the velocity of the particles by laser Doppler velocimetry under an electric field, employing a Zetasizer Nano (Malvern Instruments), taking the average of at least 10 measurements.

Synthesis of latex protein complexes

LPC were obtained by physical adsorption of TBH onto polystyrene latex S8, S10 and S13 and by covalent coupling of TBH onto carboxylated latexes (S8C1, S8C2, S10C1, S10C2, S13C1 and S13C2).

In the physical adsorption experiments, a volume of each latex which corresponded to an area of 0.2 m^2 was mixed with TBH at concentrations of 0.9 mg/mL and 2 mg/mL (C°). The reaction was carried out at low ionic strength (0.002 M) in phosphate buffer (pH 5), maintaining the mixture under stirring during 5 h at room temperature. After incubation, the latex–protein complexes were centrifuged for 30 min at 15,000 rpm and the sensitized particles were resuspended in borate buffer (pH 8) and kept at 4 °C. The adsorbed protein was determined from the difference between C° and the protein remaining in solution (C_{sol}) by the bicinchoninic acid method.

In the covalent coupling experiments onto carboxylated latexes, a volume of each latex which corresponded to an area of 0.2 m^2 was mixed with TBH at C° = 0.9 and 2 mg/mL, in the presence of the EDC activator. The activation of carboxyl groups was performed simultaneously with the covalent coupling to minimize the hydrolysis of the acylurea intermediate. Incubation in phosphate buffer (pH 5) was carried out under stirring for 5 h at room temperature. The resulting latex–protein complexes were first isolated from the solution by ultracentrifugation during 30 min at 8000 rpm and then resuspended in Triton X-100 1% (v/v) for 24 h to desorb proteins not covalently attached to the particles. Once again, the latex–protein complexes were isolated from the solution by ultracentrifugation, resuspended in 0.002 M borate buffer (pH 8) and kept at 4 °C. The total-linked protein (i.e., both physically adsorbed and covalently bound) and the covalently-coupled protein (i.e., the protein that remained on the particle surface after desorption with Triton X-100) were determined through a mass balance.

A refrigerated centrifuge Neofuge 18R (Heal Force) and an UV–Vis spectrophotometer Lambda 25 (PerkinElmer) were used for protein separation and quantification, respectively.

Immunoagglutination assays

The agglutination test was performed on a dark background agglutination card. According to Garcia et al. (2017), the latex protein complexes were centrifuged and resuspended in a buffer containing glycine, polyethylene glycol and bovine serum albumin, in order to obtain a final concentration of 0.5% solids. Later 40 µL of latex protein complex and 40 µL of control positive serum (or control negative serum) were mixed, registering the presence or not of agglutination at 5 min. Two positive and 2 negative sera were employed.

Results and discussion

Synthesis and characterization of polystyrene latex particles

Table 1 shows the final values of conversion, particle diameter and polydispersity index achieved in each experiment. In addition, SEM and TEM images are included as supplementary material.

A MA-80 concentration below its CMC was used for producing the S8 latex, but concentrations higher than the CMC were utilized for synthesizing S9 and S10 latexes, thus ensuring micelle formation. Changes in the Sty/MA-80 mass ratio allowed obtaining latexes with varied D_{DLS}: 85 nm (S9), 130 nm (S10) and 300 nm (S8).

The evolutions of conversion and average particle diameter as a function of reaction time in emulsion polymerization are shown in Fig. 1. In all cases, conversions rapidly increased and their final values were close to 100%. After 2 h of reaction the conversions reached for latexes S8, S9 and S10 were 98.4%, 99.9% and 98.8%, respectively. Latexes S8 and S10 reached the maximum conversion at 60 min and then remained constant, while for latex S9 the maximum conversion was reached at 40 min (Fig. 1). These observations are explained by the presence of a significant concentration of Em in the synthesis of S9, which increases the micellar nucleation rate (and produces smaller particle diameters), giving rise to high reaction rates. Note also that D_{DLS} grows in the course of the reaction, in agreement with the monomer conversion, and the maximum D_{DLS} value was observed at the same time as the highest conversion.

Figure 2 shows the effect of MA-80 concentration on the final particle number for latexes S8, S9 and S10, at a fixed $K_2S_2O_4$ concentration (see Table 1). Based on the expected linear relationship between N_p and $[MA\text{-}80]^{0.6}$ predicted by

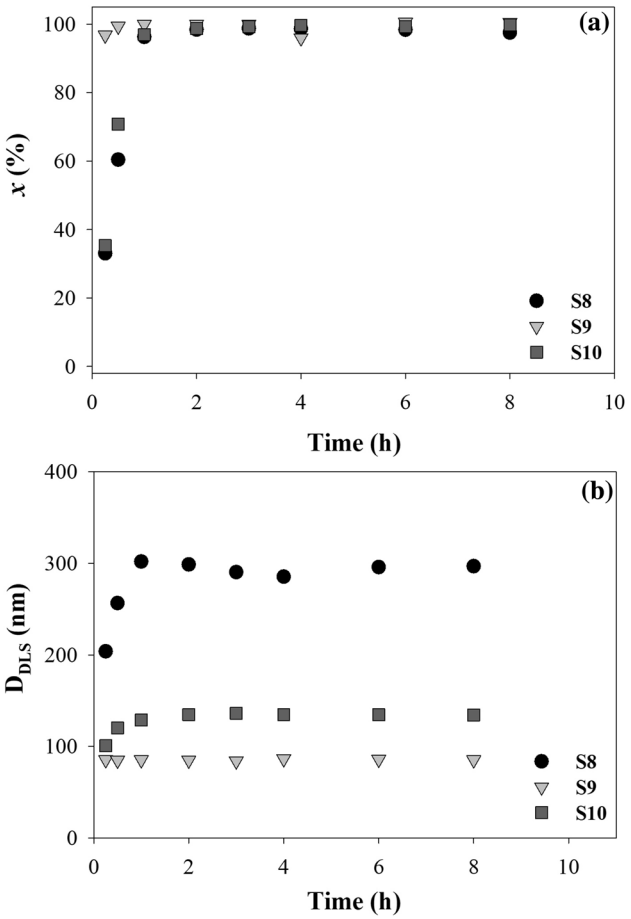

Fig. 1 Time evolution of the monomer conversion (**a**) and the average particle diameter measured by DLS at 90° (**b**) for the synthesis by emulsion polymerization of PS latexes S8, S9 and S10

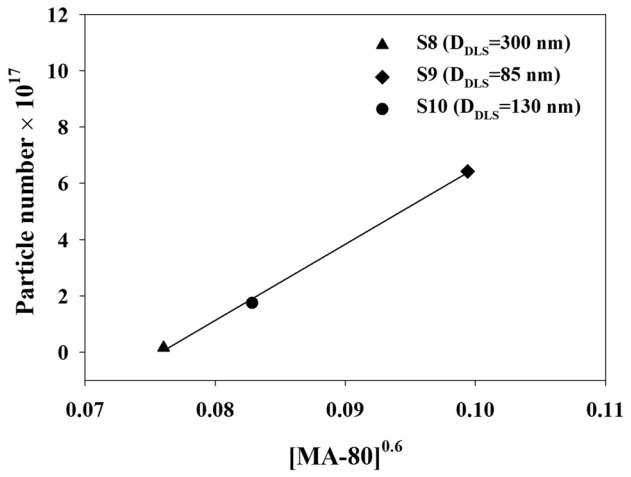

Fig. 2 Influence of the MA-80 concentration on the number of particles in the styrene emulsion polymerization for producing PS latexes S8 (D_{DLS} = 300 nm), S9 (D_{DLS} = 85 nm) and S10 (D_{DLS} = 135 nm)

the Smith-Ewart theory, the [*Em*] necessary to obtain the desired particle diameter for the synthesis of latex S10 was previously established.

The evolution of conversion and average particle diameter as a function of reaction time obtained in dispersion polymerization are presented in Fig. 3. Syntheses of latexes S11 and S12 reached final conversions of 67% and 70%, respectively. Note that when experiment S11 was stopped, the monomer conversion was still growing at a high reaction rate. In both experiments, S11 and S12, $D_{DLS} > 2$ µm (not reported because they exceed the range of the DLS equipment utilized). The dispersions of both beads proved to be unstable because of their sizes, which were determined by TEM (S11 = 2.90 µm and S12 = 1.74 µm).

Experiments S13, S14, S16 and S17 produced final conversions above 95%. It was also noted that the ethyl alcohol/water mixture in a ratio 2.2:1 was the most suitable dispersion medium to obtain stable latexes with particle diameters between 500 and 1050 nm.

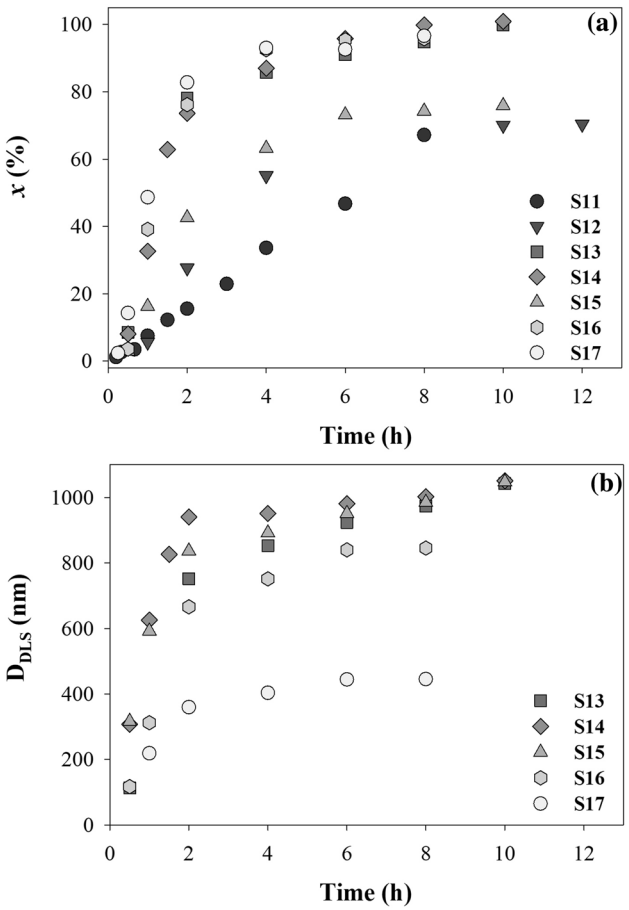

Fig. 3 Time evolution of monomer conversion (**a**) and average particle diameter measured by DLS at 90° (**b**) for the synthesis by dispersion polymerization of PS latexes S11, S12, S13, S14, S15, S16 and S17

Concentrations of PVP, AIBN and Sty were varied in experiments S13, S14, S15, S16 and S17 and it was observed that: (1) the reduction in the amount of the initial monomer (see columns S14 vs. S15 in Table 1) gave place to lower values of reaction rates, final conversion, and polydispersity index, without practically affecting the final D_{DLS}, (2) the increase of PVP concentration (see columns S13 and S16) reduced the particle diameter and this effect was higher when the AIBN concentration was decreased (column S17). This last observation is in accordance with results by Cho et al. (2016), where the effect of reactants (Sty, AIBN and PVP) and conditions (temperature, dispersion medium) were modified to control the particle diameter in dispersion polymerization. When the radical concentration of AIBN in monomer droplets increased, it resulted in the enlargement of the particle diameter as a function of polymerization temperature. In this work, we observed that particles with smaller size were obtained when AIBN concentration was reduced, since at the same temperature, the radical concentration in monomer droplets decreased.

Synthesis and characterization of functionalized latex particles

The evolution of conversion and average particle diameter as a function of reaction time are shown in Fig. 4 for the synthesis of: (1) six carboxylated latexes, and (2) two particle dispersions with epoxy functionality.

Final conversion for experiment S8C1 was around 75%. After a second load of initiator in experiment S8C2, an increment in the final conversion was observed, reaching a value of 86%. The final conversions for experiments S10C1 and S10C2 were lower than 60%, and this could be due to the formation of a significant amount of coagulum, which was not quantified. However, for S13C1 and S13C2 final conversions higher than 90% were obtained. Notice that for all carboxylated latexes, the incorporation of functional groups was achieved without significantly changing the average particle diameter nor producing a secondary particle population (Tables 1, 2). For this reason, polydispersities of the carboxylated latexes were similar to those of the corresponding PS seeds.

Latexes with epoxy functionality (S8E1 and S8E2) reached final conversions of 69% and 80%, respectively. The final D_{DLS} obtained for latex S8E1 was about 20% higher

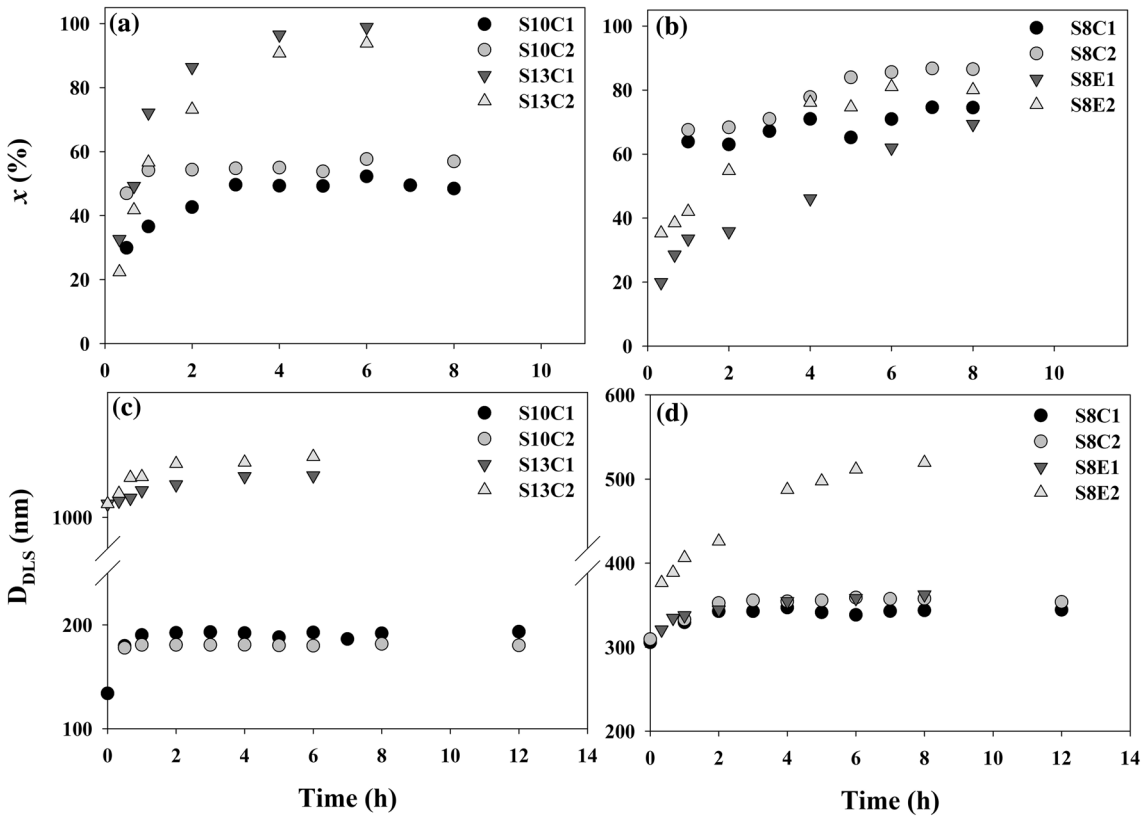

Fig. 4 Evolution of monomer conversion (**a**, **b**), and average particle diameter measured by DLS at 90° (**c**, **d**) as a function of reaction time in the synthesis of carboxylated latexes S8C1, S8C2, S10C1, S10C2, S13C1 and S13C2; and epoxylated latexes S8E1 and S8E2

than that of the base latex seed S8 (D_{DLS} = 300 nm), achieving the objective of incorporating epoxy functional groups with a polydispersity lower than 1.02. However, the final D_{DLS} and PI of latex S8E2 were higher than expected, and this could be due to problems associated with the synthesis process, such as poor agitation, presence of O_2, insufficient swelling of the particles and/or the presence of agglomerates or clots in the PS seed.

Synthesis of latex protein complexes

Results of LPC synthesis are shown in Fig. 5. The sensitization experiments were carried out at pH 5, which was the better condition previously studied by Prochetto et al. (2017) for this antigenic mixture and similar latexes. Experiments were performed at two initial concentrations of TBH (C°) in order to obtain different amounts of bound protein. As expected, the higher the initial protein concentration, the greater the protein amount obtained on the particle surface. In addition, in covalent coupling experiments with carboxylated latexes produced on the basis of the same seed, it was observed that the higher the density of carboxyl functional groups (S8C2 vs S8C1, S10C1 vs S10C2 and S13C2 vs S13C1), the greater the amount of protein coupled.

Regarding the particle size, latexes of small diameters (S8 and S10 series) yielded similar values of bound protein; while for latexes of large diameters (S13 series), the amount of protein coupled was lower. These results are in agreement with those observed in sensitization experiments of this kind of latexes with proteins of *Toxoplasma gondii* (Peretti 2015).

Immunoagglutination assays with visual detection

The 18 LPC obtained with TBH and different latexes (Fig. 5) were analyzed in immunoagglutination assays against 2 positive control and 2 negative control bovine serum samples.

The LPC obtained from the S8 and S10 seeds did not present any agglutination. Many variables in addition to size influence the formation of the agglutination network and its visual detection, such as the concentration of particles in the test, the amount of protein bound to the particles, dilutions of the samples used and the formulation of the reaction buffer. According to Molina-Bolívar and Galisteo-González (2005) many of the latex agglutination tests developed are performed manually and the agglutination is detected by visual observation. In these tests, larger particles of several hundred nanometers seem to be more appropriate, the most common size being 800 nm. Although in some situations good performance can be observed in the immunoagglutination assay with smaller particles, in the conditions tested in this work this did not occur.

With carboxylated latex–protein complexes S13C1 and S13C2, agglutination was not observed when LPC were tested with control positive serum. A possible explanation for this result is that the surface charge of the latexes under the conditions employed in the experiments maintained the

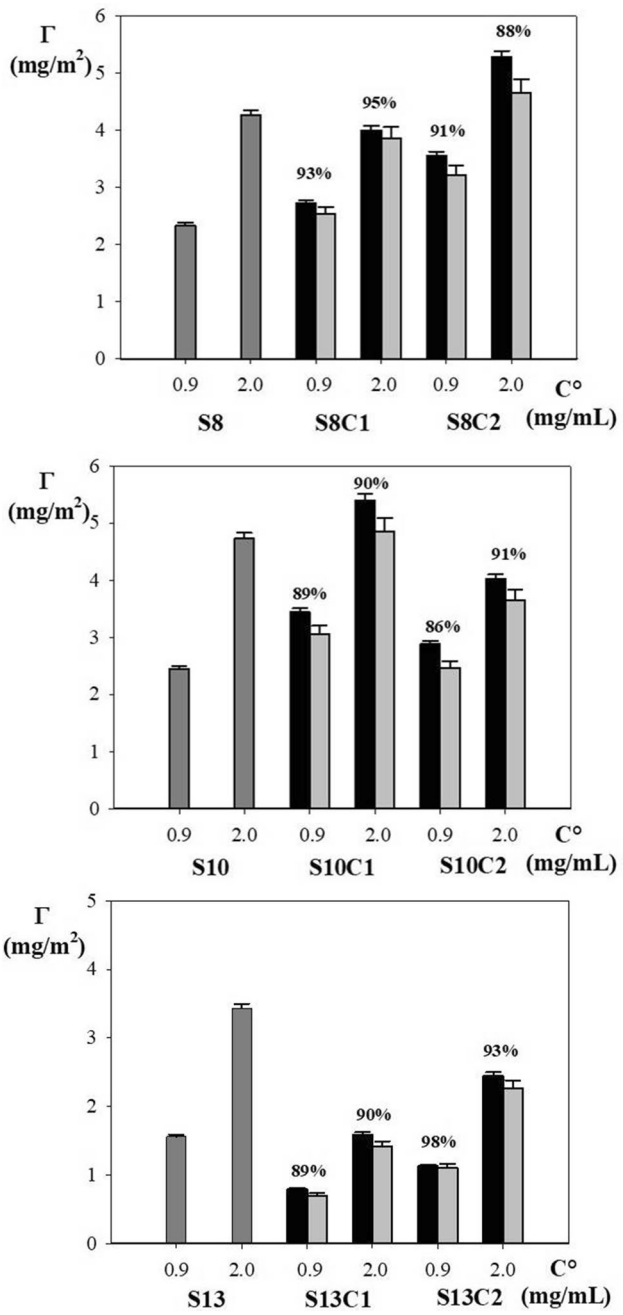

Fig. 5 Physical adsorption of TBH onto latexes S8, S10 and S13 and chemical coupling onto the carboxylated latexes S8C1, S8C2, S10C1, S10C2, S13C1 and S13C2, as a function of the initial protein concentration, at pH 5. The surface density of total linked protein (black) onto carboxylated latexes is compared with the covalently coupled protein (grey), after desorption with Triton X-100. The percentages on the grey bars indicate the fractions of covalently coupled protein and error bars show the standard deviations for three replicates

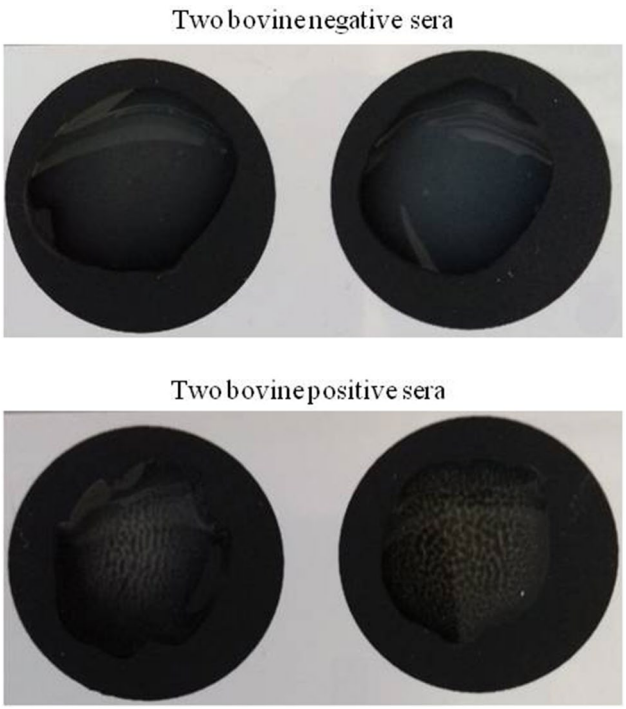

Fig. 6 Latex agglutination test with visual detection after 5 min of reaction. Latex protein complexes were obtained with TBH and polystyrene latex S13 ($\Gamma = 3.43$ mg/m^2)

system stable, thus avoiding the agglutination in spite of the presence of specific Ab.

Finally, good results were reached with the LPC obtained from the PS latex S13. Both LPC, with $\Gamma = 1.56$ mg/m^2 and $\Gamma = 3.43$ mg/m^2, showed a good discrimination between positive and negative sera. It was observed that, at 5 min of reaction, discrimination between agglutinated and not agglutinated LPC was clear. Photographs of the latex agglutination test performed are shown in Fig. 6.

Conclusions

PS particles of varied size were produced using both emulsion and dispersion polymerization. In the EP experiments for the synthesis of PS latexes S8, S9 and S10, high conversions (greater than 98%) and different particle diameters (in the range 85–300 nm) were achieved, depending on the emulsifier concentration (MA-80) employed, resulting in PSD with polydispersities lower than 1.01. In the DP experiments for the synthesis of PS latexes S13 and S14, conversions close to 100% were obtained, and above 95% for S16 and S17; whereas in the production of PS latexes S11, S12 and S15 lower conversions (between 67 and 75%) were achieved. Also, the increase in PVP and the decrease in AIBN concentrations gave a reduction in the final particle diameter and in the polydispersity index (S17).

In the synthesis of functionalized particles with core–shell morphology by seeded emulsion polymerization, low conversions (between 49 and 86%) were generally achieved, except for carboxylated latexes S13C1 and S13C2 where final conversions were 99% and 94%, respectively. In addition, the variation of the monomer ratios, MAA/Sty and GMA/Sty, allowed modifying the density of surface functional groups of the polymer particles.

LPC were produced by physical adsorption of the TBH antigen onto the synthesized PS latexes, and by covalent coupling of this antigen onto carboxylated latexes, thus finally producing complexes of varied characteristics.

The LPC produced from the S13 latex (LPC S13-TBH) was tested against a couple of control (positive and negative) sera; and it was shown that, under the experimental conditions investigated here, the LPC S13-TBH (with $\Gamma = 1.56$ mg/m^2 and $\Gamma = 3.43$ mg/m^2) are suitable as LAT reagents for detecting leptospirosis in bovine sera, by visual detection.

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