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# Interfacial deposition of functionalized copolymers onto nanoemulsions produced by the solvent displacement method

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**Abstract** Cationic nanoemulsions containing an oily core as potential carriers of nucleic acids were prepared by a solvent displacement method in the presence of a nonionic surfactant (Pluronic F68). With a view to functionalize such nanoemulsions for further incorporation of a fusogenic peptide, a poly(maleic anhydride-*alt*-methyl vinyl ether) ($M_n = 67{,}000$) grafted with variable amount of acetylspermine (or acetylspermidine) and decylamine was nanodeposited during the nanoemulsion formation step. Functionalized nanoemulsions were characterized in terms of particle size (by quasi-elastic light scattering and electron microscopy), electrophoretic mobility and long-term stability as a function of the amount of polymer used in the formulation. It was found that increases in the level of the copolymer led to a reduction in the particle size and a decrease in colloidal stability. In addition, the incorporation of the grafted copolymers at the interface of the nanoemulsions was clearly evidenced, a shift towards low pH at the point of zero charge being attributed to the formation of carboxylic groups induced by hydrolysis of the residual maleic anhydride groups of the copolymer.

**Key words** Nanoemulsions · Functionalized copolymers · Interfacial deposition

## Introduction

Colloidal carriers are potential substitutes to viral vectors in gene therapy. The nucleic acids can be either adsorbed on the surface [1, 2] or encapsulated inside the colloid, for example, after oligonucleotide hydrophobization as described by Berton et al. [3] for PLA nanoparticles or more recently by an encapsulation process involving interfacial polymerization [4].

Nevertheless, these two approaches lack the potential of binding, after the nucleic acid incorporation step, further molecules, such as a fusogenic peptide [5], to enhance the release from endocytic vesicles or a proper molecule to achieve cell targeting (mannose, for instance, to target dendritic cells).

Our approach to address this issue was based on our recent results on the binding of peptides onto functional polymers [6]. Our strategy was to achieve the surface functionalization of colloidal carriers with a polymer bearing functional groups capable of covalently reacting with a fusogenic peptide.

Among all the potential colloidal carriers in the submicronic range, various types of dispersed systems can be achieved by a solvent displacement process: nanoemulsions (NEs) (oily core), nanospheres (where the core of the particle is a polymer) or nanocapsules (where an oily core is surrounded by a rigid polymeric membrane) [7, 8, 9]. We selected the NEs on the basis of previous investigation by Teixeira et al. [1], who obtained them by the solvent displacement method. The surface functionalization of the emulsions was expected to take place during the solvent displacement step. Therefore, the polymer was designed as follows: aliphatic arms to anchor the hydrophilic polymer at the NE interface by hydrophobic interactions with the oily core of the capsule; cationic charges to allow the

complexation of the nucleic acids and potential functional groups for subsequent peptide binding.

This article reports preliminary results on the derivatization of polymers to meet all the previously mentioned criteria, on the interfacial deposition of the polymer and finally on the effect of the adsorbed polymer on the colloidal stability of the functionalized NEs.

## Materials and methods

### Materials

The poly(maleic anhydride-*alt*-methyl vinyl ether) copolymer ($M_n$: 67,000) [P(MAMVE)] was from Polysciences (Warrington). Decylamine was obtained from Aldrich (Steinheim), $N^8$-acetylspermidine dihydrochloride and acetylspermine trihydrochloride salts were supplied from Fluka (Steinheim). NEs were prepared using medium-chain triglyceride (MCT) as an oily core (Société des Oléagineux, St. Laurent, Blangy, France), lipoid E-80, containing mainly phosphatidylcholine (85%) and phosphatidylethanolamine (8%) (Lipoid, Ludwigshafen, Germany), poly(oxyethylene)–poly(oxypropylene)–poly(oxyethylene) triblock copolymer Pluronic F68 (BASF, Ludwigshafen, Germany) and stearylamine (SA, Sigma, Mo., USA). Anhydrous dimethyl sulfoxide (DMSO) and all other solvents, of analytical grade, were from Aldrich.

### Synthesis and characterization of functionalized MAMVE copolymers

Copolymer A (66% $N^8$-acetylspermidine and 45% decylamine with respect to total anhydride functionality) was synthesized as follows. $N^8$-acetylspermidine dihydrochloride (100 mg, 0.384 mmol, $M = 260.21$) in solution in 6 ml anhydrous DMSO with an equimolar amount of triethylamine, was added to P(MAMVE) (90 mg, 0.578 mmol anhydride functions), also solubilized in anhydrous DMSO (6 ml). After 20 h of reaction at room temperature, 52 $\mu$l (0.26 mmol) decylamine ($M = 157.3$, $d = 0.787$) was added to the mixture. The resulting functionalized copolymer was precipitated in diethyl ether and solubilized in water. This solubilization was facilitated by adding 4 M HCl (50 $\mu$l). The polymer was dialyzed against water and lyophilized. The total reaction yield was about 70%.

Copolymer B (33% acetylspermine and 45% decylamine with respect to total anhydride functionality) was synthesized following the procedure described for copolymer A. Acetylspermine trihydrochloride (68 mg, 0.192 mmol, $M = 353.76$) substitued the amount of acetylspermidine used in the reaction (total reaction yield: 70%). The excess of anhydride moieties were hydrolyzed during the dialysis step.

The copolymers obtained were characterized by $^1$H NMR spectroscopy (Varian 500 MHz) and fluorimetry (Perkin Elmer LS50) with the use of fluorescamine for the titration of residual primary amines in the reaction mixture. This method allows one to follow the kinetics of the reaction. For this purpose, at defined times, 20 $\mu$l was taken from the reaction mixture and added to an excess of fluorescamine (solubilized in 300 $\mu$l DMSO). After 30 min of storage in the dark, the sample was diluted 20-fold in DMSO for the fluorimetry analysis ($\lambda_{exc} = 420$ nm, $\lambda_{em} = 477$ nm). The fluorescence intensity obtained was related to the concentration of residual amines ($N^8$-acetylspermidine dihydrochloride, acetylspermine trihydrochloride or decylamine) using a calibration curve $I_{fluo} = f([N^8\text{-acetylspermidine dihydrochloride}], [\text{acetylspermine trihydrochloride}] \text{ or } [\text{decylamine}])$ established under the same conditions. Therefore, the conversion could be determined provided the initial amine concentration was known.

### Preparation of NEs

NEs were prepared by a modification of the method described by Fessi et al. [9], based on interfacial deposition of preformed polymers following solvent displacement. Typically, MCT (375 $\mu$l), lipoid E-80 (100 mg) and SA (25 mg) were dissolved in 2 ml ethanol. This solution was then added to a solution of acetone/water (10.4 ml/2.6 ml) containing various amounts of functionalized MAMVE copolymer and 10 $\mu$l trifluoacetic acid (TFA). Then, this organic solution was poured, under moderate magnetic stirring, into a 30 ml aqueous phase containing 84 mg nonionic surfactant, Pluronic F68. The resulting mixed phase immediately turned milky as a result of the formation of NEs. After stirring for 1 h, organic solvents and a part of the water were removed under reduced pressure at 40 °C to reach a final volume of about 5 ml. The pH of the emulsions obtained was acidic (2–3) owing to the presence of TFA.

### Stability and mean diameter of the NEs

The stability with storage in terms of the mean particle size was evaluated for NEs synthesized with functionalized copolymers. The mean size of the NEs was determined in $10^{-3}$ M NaCl, at 20 °C, by quasi-elastic light scattering measurements using a Zetasizer 3000 HS (Malvern Instruments). The NEs were considered to be destabilized when the mean diameter measured was increased by 20% with respect to initial values.

### Pluronic F68 and copolymer quantification in the aqueous phase of NE preparations

Ultrafiltration was performed on NEs (0.1 $\mu$m cutoff, Ultrafree MC Millipore, Bedford, USA.). The ultrafiltrate obtained (volume $v$) was added to a solution of trioxane in $D_2O$ (volume $v_t$, concentration $c_t$) and analyzed by $^1$H NMR spectroscopy (200 MHz, Bruker).

The total amount of nonionic surfactant Pluronic F68, HO–$(CH_2CH_2O)_{76}$–$(CH_2CH(CH_3)O)_{29}$–$(CH_2CH_2O)_{76}$–H, in the aqueous phase of the NE preparations (volume $V$) could be estimated by the following equation:

$$m_p = \left(c_t v_t \times \frac{I_p}{I_t} \times \frac{6}{695}\right) \times M_n \times \frac{V}{v} \quad .$$

$I_t$ is the peak integral of the $-CH_2-$ groups of the trioxane ($\delta = 5.2$ ppm) (corresponding to six protons), $I_P$ is the peak integral of the $-CH_2-$ groups of the Pluronic ($\delta = 3.4$–$3.75$ ppm) (corresponding to 695 protons) and $M_n$ is the molecular weight of Pluronic (8350 g/mol).

The relative amount of copolymer A in the aqueous phase was determined by comparing the peak integral of the methyl ($N^8$-acetylspermidine dihydrochloride) group of the ultrafiltrate NMR spectrum ($I_{ult}$) with that detected in the emulsion spectrum ($I_e$), using an internal standard (whose integral was calibrated to a value of 1). The Pluronic of the emulsion could be used as the standard since it was shown to be entirely located in the continuous phase. The peak integral of the $-CH_3$ groups of Pluronic (1.14 ppm) was used as a reference for the spectra of both the emulsion and the ultrafiltrate. The ratio $I_{ult}/I_e$ allowed one to estimate the percentage of polymer in solution.

### Surface characterization of NEs

The determination of the electrophoretic mobility was performed by the technique of laser Doppler anemometry using a Zetasizer 3000HS (Malvern). The curves of the zeta potential versus the pH were established (Malvern instrument equipped by a Mettler titrator) in order to clarify the nature of the surface charge of

the particles. The titration of an alkaline solution ($10^{-3}$ M NaOH) was performed by addition of $10^{-2}$ M HCl aqueous solution.

### Morphology of the NEs

The morphology of the NEs was examined by the freeze-fracture microscopy technique (FC150 Reichert unit) using the following procedure. A droplet of the sample was deposited on a small copper cup and was rapidly frozen in a nitrogen slurry (–210 °C). After shadowing the freeze-fractured surfaces with platinium (2 nm), the samples were coated with carbon (8–10 nm). This process was carried out at –150 °C and under reduced pressure (below $10^{-5}$ torr). The replicas were allowed to warm to room temperature and were hardened with collodion, washed in a mixture of nitric acid, phosphoric acid, and acetic acid (1:1:1, volume ratio), rinsed with distilled water, and washed again with a sodium hypochloric solution. A last washing was performed with ethanol (50 v% in water) and methanol (50 v% in water) to completely remove potential traces of the initial product. After mounting on a copper grid and removal of the collodion with isoamyl acetate, the replicas were examined by transmission electron microscopy (TEM, Philips CM120).

The observation of the NEs was also performed by the scanning electron microscopy (SEM) technique. The preparation of the samples consisted of vitrifying a film of the emulsion by plunging it into liquid nitrogen. The sample was then metallized with gold and was observed using an S800 Hitachi scanning electron microscope.

## Results and discussion

### Polymer design

Preliminary experiments showed that no stable emulsion could be obtained using P(MAMVE) in the hydrolyzed form (Fig. 1a). The use of the unhydrolyzed copolymer (Fig. 1b) led to an emulsion with a poor shelf life, probably owing to the hydrolysis by water molecules of the anhydride moities to carboxylate groups, which drastically increased the water solubility of the polymer. Therefore, to obtain stable and cationic emulsions, the copolymer was modified with hydrophobic arms to anchor the polymer in the oily core of the NE and spermine or spermidine arms to bring some cationic charges.

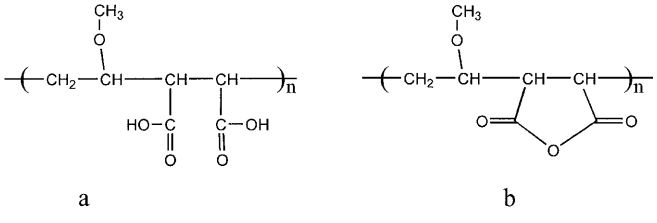


**Fig. 1** Structure of **a** the hydrolyzed form and **b** the unhydrolyzed form of poly(maleic anhydride-*alt*-methyl vinyl ether) [*P(MAMVE)*] ($n = 430$, $M_n = 67000$ g/mol)

### Characterization of copolymers A and B

The reaction kinetics between amine components (acetylspermidine/acetylspermine and decylamine) and P(MAMVE) were investigated for copolymers A and B (Fig. 2). Conversions were determined by fluorimetry as described in the Materials and methods. At different times of the reaction, the free amine in solution was quantified via specific reaction with fluorescamine. Preliminary studies showed that DMSO, P(MAMVE), triethylamine and diisopropylamine (secondary amine) did not lead to a fluorescence signal in the presence of fluorescamine. The fluorescence intensity with fluorescamine was only observed for acetylspermidine, acetylspermine and decylamine, which confirmed that the reaction was primarily amine-specific (Fig. 3).

The results obtained for copolymer B synthesis are shown in Fig. 4. Acetylspermine was first reacted with P(MAMVE) for 20 h and the coupling reaction was monitored by the fluorescence (Fig. 4a). In a second step, decylamine was introduced to the solution and the coupling was checked as described earlier (Fig. 4b). Concerning the coupling of decylamine, it was necessary to subtract the intensity due to the residual acetylspermine in solution from the values of the fluorescence intensity obtained.

As a result from both coupling kinetics, a reaction time around 20 h appeared to be sufficient. The same result was obtained from copolymer A synthesis.

The functionalization rates were calculated from the following general equation:

$$f_{\text{amine}} = n_{\text{amine}} \times c_{\text{amine}} \quad .$$

$f$ is the functionalization rate of the amine on P(MAMVE), $n$ is the equivalent number of amines per anhydride function and $c$ is the final conversion of the functionalization reaction between the amine and P(MAMVE) after 20 h.

The functionalization results are reported in Table 1. The acetylspermidine coupling reaction yield was similar to that observed with acetylspermine (conversion: 84 and 76%, respectively) even if the molar equivalent of reactant per anhydride function was different (0.66 or 0.33 Eq).

Concerning the functionalization of copolymers with decylamine, the coupling yield obtained for copolymer B was higher ($f_{\text{dec}} = 0.40$) than for copolymer A ($f_{\text{dec}} = 0.31$). This can be explained by steric hindrance, which is much more significant for copolymer A. Indeed, when decylamine was added to the reaction mixture, P(MAMVE) was already functionalized at 55% by acetylspermidine arms for copolymer A, whereas it was only 25% functionalized by acetylspermine arms in the case of copolymer B.

Qualitatively, $^1$H NMR 500 MHz spectra of purified copolymers A and B (Fig. 5) showed that P(MAMVE) was successfully functionalized by both

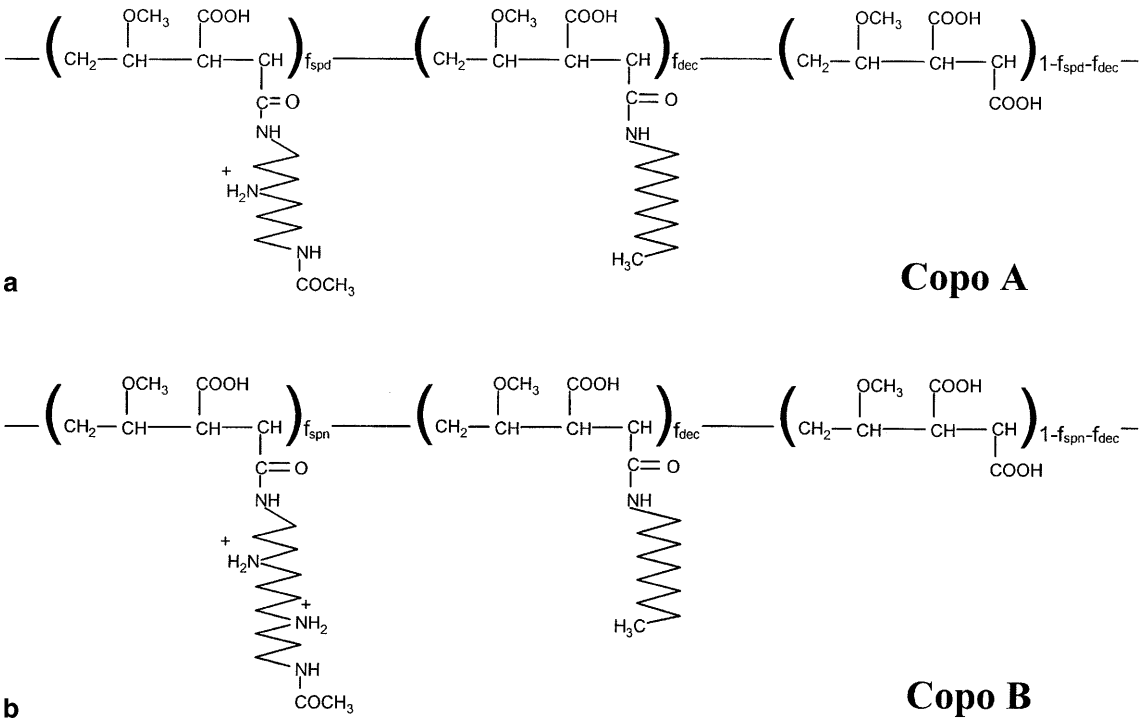


**Fig. 2** Chemical structure of **a** copolymer A and **b** copolymer B. The repartition of units is statistical. *f* represents the functionalization rate of the amine ($N^8$-acetylspermidine dihydrochloride, *spd*, acetylspermine trihydrochloride, *spn*, decylamine, *dec*) per anhydride function. The experimental values are reported in Table 1

acetylspermidine/acetylspermine and decylamine. Unfortunately, no precise functionalization rates could be determined because of peaks overlapping between P(MAMVE), acetylspermidine/acetylspermine and decylamine.

In a semiquantitative way, the $N^8$-acetylspermidine dihydrochloride/decylamine ratio (for copolymer A) and the acetylspermine trihydrochloride/decylamine ratio (for copolymer B) were determined approximately by using the $CH_3$ peak integrals of acetylspermidine or acetylspermine and the $CH_3$ peak integrals of decylamine. As shown in Table 2, the ratios obtained both by NMR and fluorimetry were similar.

## NE synthesis and stability studies[1]

The NEs were successfully synthesized in the presence of copolymers A and B (NEA and NEB, respectively). On the basis of the modification of the P(MAMVE) copolymer, it was expected to obtain NEs with an interfacial layer containing copolymers A or B. The method required the copolymer to be dissolved in acetone; however, since copolymers A and B were not soluble in pure acetone, it was necessary to dissolve them first in water in the presence of TFA and then to add acetone (volume ratio acetone:water 4:1).

NEs synthesized with copolymers A and B (50 mg in the formulation) ($NEA_{50}$ and $NEB_{50}$) revealed quite good colloidal stability (Table 3): however, the stability was better for $NEB_{50}$ than for $NEA_{50}$ (45 and 10 days, respectively). Indeed, the mean size obtained for $NEB_{50}$ was found to be smaller than for $NEA_{50}$. Thus, the structure and composition of copolymer B seemed to be more efficient at ensuring the colloidal stability of the NEs and consequently at decreasing their size (NEs prepared without copolymer, $NE_0$, exhibit an average size of 200 nm).

Conversely, NEs could not be obtained with copolymer $A_{no\ dec}$ [P(MAMVE) functionalized with 66% of acetylspermidine]: during solvent evaporation under reduced pressure, a phase separation took place. NEs could be synthesized with copolymer D [P(MAMVE) functionalized with 45% of decylamine] but they revealed poor stability, with aggregation occurring very

[1]Despite the similarities between the process used here and that currently used for nanocapsule formation [9, 10], the nanocapsule term is not appropriate for the objects investigated in this work. Indeed, the modified P(MAMVE) copolymers used alone (i.e., without the oil phase) were found not to lead to the formation of nanospheres (the copolymers are soluble in water at acidic pH), whereas such nanospheres are systematically produced in the case of the polymers typically used for nanocapsule formulation [poly(ε-caprolactone), poly(D,L-lactide)]. The dispersed systems described here are much better defined as being NEs functionalized at their surface by a copolymer containing hydrophobic arms which ensure anchoring at the interface

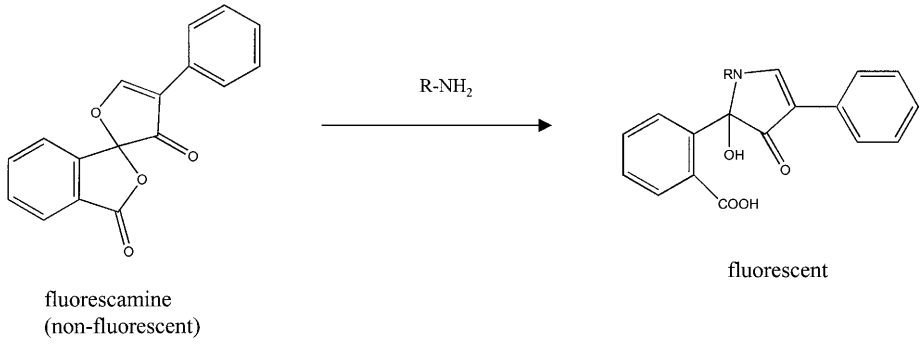


**Fig. 3** Scheme of the reaction between fluorescamine and primary amine

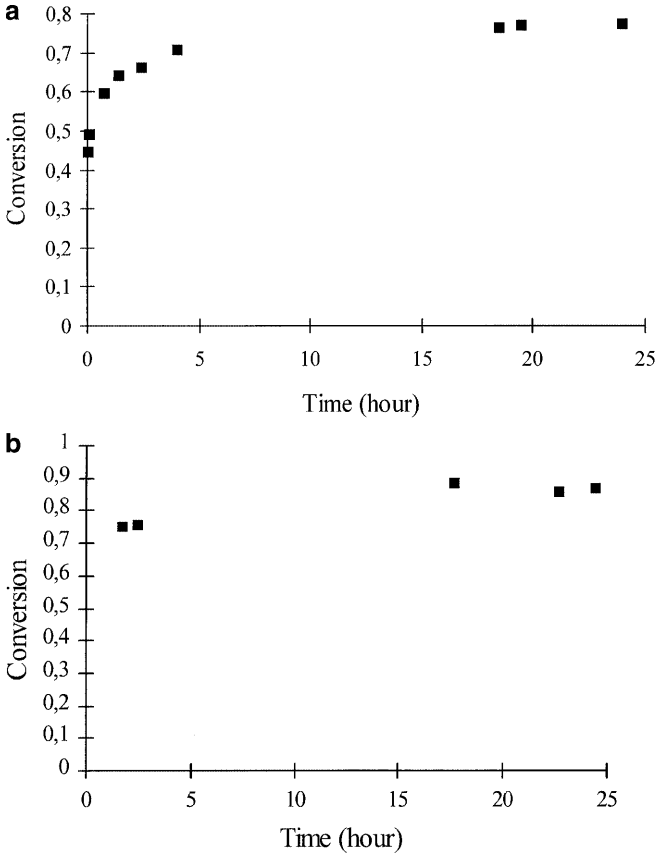


**Fig. 4** Kinetics study of **a** the reaction between acetylspermine and P(MAMVE) and **b** the reaction between decylamine and P(MAMVE) (case of copolymer B)

quickly. These results clearly showed that the preparation of stable NEs required the design of a modified P(MAMVE) having both cationic (acetylspermidine or acetylspermine) and hydrophobic arms.

## Pluronic F68 and functionalized copolymer location

$^1$H NMR spectra of ultrafiltrates of NEs showed the presence in the liquid phase of Pluronic F68 as the major component. The data obtained for experiments run with the NEs synthesized with copolymer A (50 mg in the formulation) are reported in Table 4. The $^1$H NMR analysis of the aqueous phase of NE dispersions prepared with 84 mg Pluronic F68 (standard formulation: $NEA_{50\ plu}$) showed that about 96% of the Pluronic was located in the aqueous phase. Similarly, NEs with only 10% (8.4 mg) of the standard amount of Pluronic ($NEA_{50\ plu/10}$) revealed that the quasi totality of this compound (95%) was still in the aqueous phase.

Moreover, zeta-potential measurements on both $NEA_{50}$ with Pluronic and $NEA_{50}$ without Pluronic gave the same values (50 mV), indicating there was no screening of surface charges by Pluronic, thus confirming that most of it was not located at the surface of the NEs, but essentially in the aqueous phase.

Similar results concerning Pluronic location were obtained for the NEs prepared with copolymer B. A tentative explanation of such a result is that Pluronic would probably confer colloidal stability to the dispersed system (if any) more by a depletion than by a steric mechanism [11].

The $^1$H NMR analysis of the supernatants also revealed the presence of weak peaks corresponding to the modified copolymer (particularly one of $-CH_3$ of acetylspermidine of copolymer A), indicating that a part of it was also located in solution.

The relative amount of polymer in solution could be determined in the case of NEs with 50 mg copolymer A, as described in Materials and methods. It was shown

**Table 1** Functionalization rates of copolymers A and B: $N^8$-Acetylspermidine dihydrochloride, *spd*, acetylspermine trihydrochloride, *spn*, decylamine, *dec*)

| Copolymer | | Equivalent number of amine per anhydride functions | Reaction conversion | Functionalization rate |
|---|---|---|---|---|
| A | spd | 0.66 | 0.84 | 0.55 |
| | dec | 0.45 | 0.69 | 0.31 |
| B | spn | 0.33 | 0.76 | 0.25 |
| | dec | 0.45 | 0.88 | 0.40 |

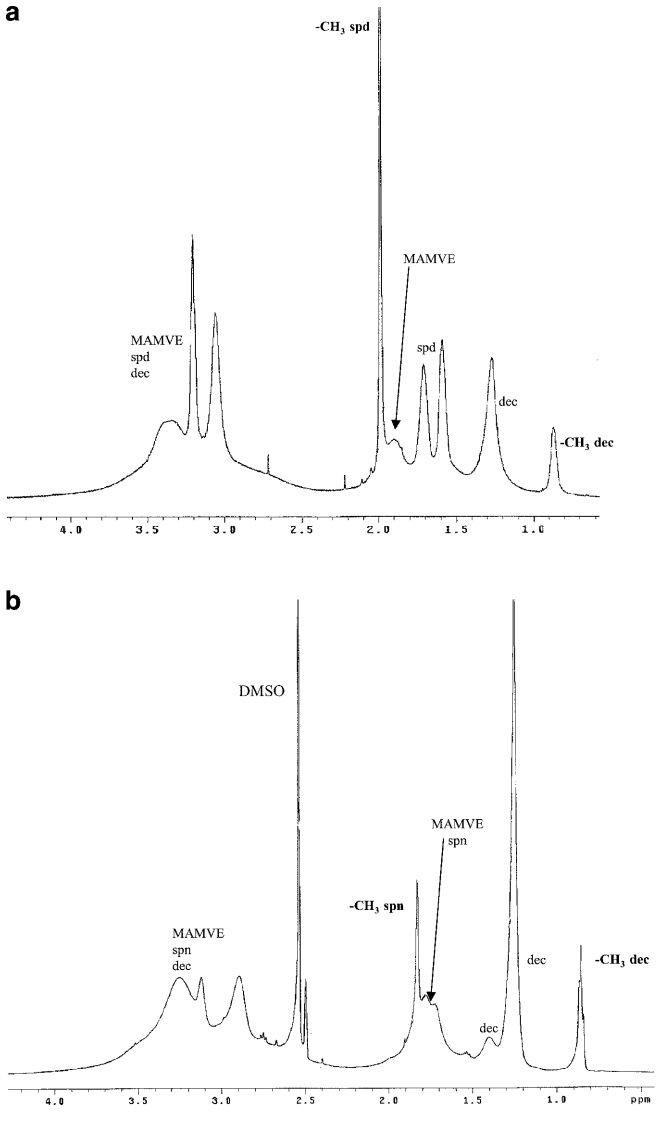


**Fig. 5** Copolymer A $^1$H NMR 500 MHz spectrum in $D_2O$ (pH 3). **b** Copolymer B $^1$H NMR 500 MHz spectrum in DMSO-$d_6$

**Table 2** Comparison between spd/dec and spn/dec ratio values obtained by $^1$H NMR and fluorimetry. spd/dec (spn/dec) is the ratio between the peak integral of $-CH_3$ spd and the peak integral of $-CH_3$ dec (ratio between the peak integral of $-CH_3$ spn and the peak integral of $-CH_3$ dec)

| | Copolymer A spd/dec | Copolymer B spn/dec |
|---|---|---|
| $^1$H NMR | 1.95 | 0.57 |
| Fluorimetry | 1.77 | 0.62 |

that about 40% of the copolymer remained free in solution. Concerning the NEs produced with copolymer B, the $-CH_3$ acetylspermine trihydrochloride peak was not detected in the ultrafiltrate spectrum, suggesting that the amount of copolymer in the continuous phase was negligible (however, this result must be considered carefully because the $-CH_3$ acetylspermine trihydrochloride peak is very weak in the emulsion spectrum).

**Table 3** Influence of functionalization recipe on stability and size of the nanoemulsions (*NE*) (polydispersity index provided by quasi-elastic light scattering: $\mu_2/\Gamma^2 < 0.17$ for all measurements). The NEs were prepared with 50 mg copolymer

| | Stability | Size (nm) |
|---|---|---|
| NEA | 10 days | 195 |
| NEB | 45 days | 120 |
| $NEA_{no\ dec}$ | No NE (phase separation) | – |
| NED | Rapid aggregation | > 300 |

**Table 4** Pluronic assay in the aqueous phase of NEs. $NEA_{50\ plu}$: NEs prepared with 50 mg copolymer A and standard amount of Pluronic (84 mg). $NEA_{50\ plu/10}$: NEs prepared with 50 mg copolymer A and 10% of the standard amount of Pluronic (8.4 mg)

| NEs | Pluronic weight in the formulation (mg) | Pluronic weight in the aqueous phase (mg) | % Pluronic in the aqueous phase ($^1$H NMR) |
|---|---|---|---|
| $NEA_{50\ plu}$ | 84 | 81 | 96 |
| $NEA_{50\ plu/10}$ | 8.4 | 8 | 95 |

## Surface characterization of NEs

The zeta potential versus pH curves relative to NE preparations containing different amounts of copolymer B are shown in Fig. 6a. The curve corresponding to $NE_0$ (no copolymer B) exhibited a typical profile of surface amino functionalized particles, conferred by the presence of SA at the surface. The phospholipids (phosphatidylcholine) used in the recipe are zwitterionic for a pH range from 3 to 10 and are supposed to have no influence in terms of surface charge. When increasing the amounts of copolymer B in the NE preparations ($NEB_9$, $NEB_{25}$ and $NEB_{50}$ corresponding, respectively, to NE with 9, 25 and 50 mg copolymer B), the curves shifted towards lower zero point of charge values. This phenomenon is interpreted in terms of the increasing amounts of polymer B, at the interfaces of $NEB_9$ through $NEB_{50}$, which bears carboxylate groups originating from the hydrolysis of the anhydride moieties.

To identify the role of the carboxylate, spermine and SA moieties on the surface net charge of the NEs, a series of experiments were designed and are reported in Fig. 6b and c.

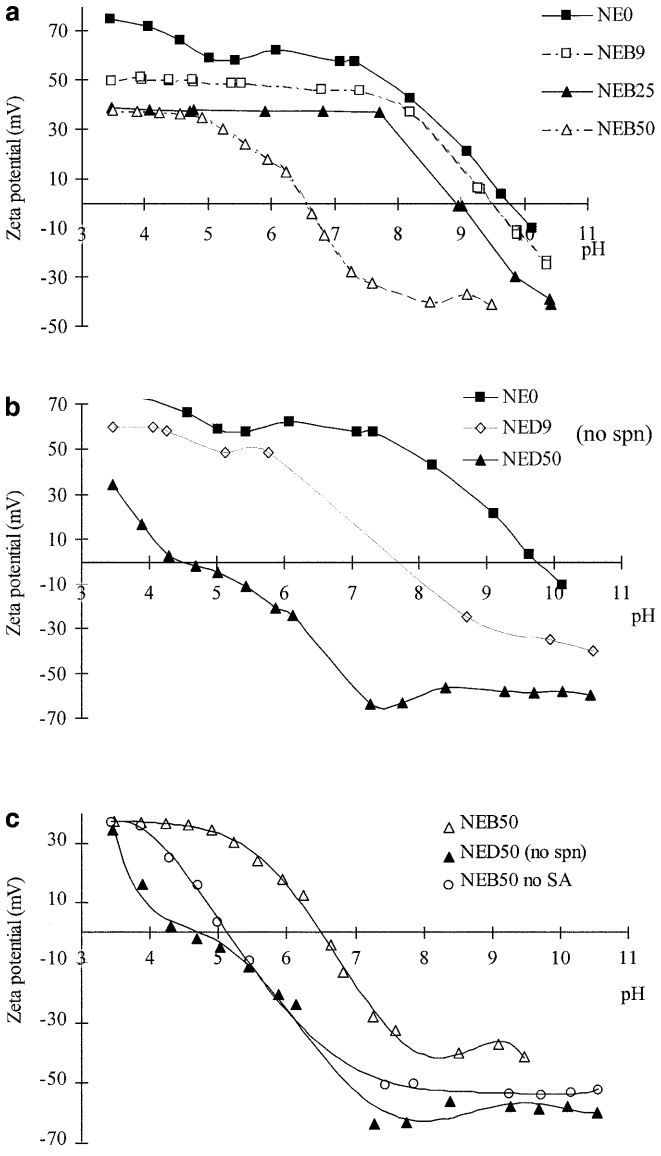


**Fig. 6** Zeta potential versus pH curves corresponding to $NE_0$ (no copolymer), $NEB_9$ (9 mg copolymer B), $NEB_{25}$ (25 mg copolymer B), $NEB_{50}$ (50 mg copolymer B). **b** Zeta potential versus pH curves corresponding to $NE_0$, $NED_9$ (9 mg copolymer D, which is devoid of spn), $NED_{50}$ (50 mg copolymer D). **c** Zeta potential versus pH curves corresponding to $NEB_{50}$, $NED_{50}$ and $NEB_{50\ no\ SA}$ (50 mg copolymer B and no SA in the formulation). The titration (Mettler titrator connected to a Malvern instrument) was started at basic pH (NaOH $10^{-3}$ M) and performed with $10^{-2}$ M HCl

First, NEs were functionalized with increasing amounts of polymer D, corresponding in composition to polymer B but lacking the acetylspermine groups. As depicted in Fig. 6b, in the presence of 50 mg polymer D in the formulation ($NED_{50}$), the zeta potential versus pH curve displays a zero net charge value of 4.6, which suggests that a polycarboxylic acid is present at the surface of the droplets of the emulsion. Therefore, all the SA present at the surface of the NE, as in $NE_0$ in which no copolymer was used, is complexed as ion pairs in $NED_{50}$, owing to the large excess of carboxylate anions brought by the copolymer. In $NED_9$, in which the amount of polymer used is 5 times lower than in $NED_{50}$, not all the stearylamines are complexed and therefore the zeta potential versus pH curve is similar to that of $NE_0$ but with a zero net charge value of 7.6, more than two pH units lower than for $NE_0$.

In the absence of SA ($NEB_{50,\ no\ SA}$, Fig. 6c), the zeta potential versus pH curve for the $NEB_{50,\ no\ SA}$ is closely related to that of $NED_{50}$, in which all the SA is complexed by the polymer. The zero net charge values are 5.2 and 4.6 for $NEB_{50,\ no\ SA}$ and $NED_{50}$, respectively. The slight difference, 0.6 pH units, can be attributed to the presence of spermine moieties in polymer B of $NEB_{50,\ no\ SA}$, which would be more oriented towards the bulk of the solution than the complexed spermine groups, despite the presence of excess carboxylate groups in the copolymer.

So, SA when used alone in the formulation confers to the NEs a positive net charge maintained until pH 9.8 (Fig. 6b); but in the presence of enough polymer, 50 mg as in $NED_{50}$, it is completely complexed and the NEs have a similar behavior as in the absence of SA ($NEB_{50,\ no\ SA}$). Knowing this, the zero net charge value of 6.5 observed for $NEB_{50}$ in Fig. 6a can be attributed to the presence of spermine groups. In other words SA participates in the anchoring of the polymer at the surface of the NE, along with the decyl groups and allows the release of the acetylspermine moieties towards the bulk for further complexation with nucleic acids. In the absence of SA, the acetylspermine would be complexed by the polymer, leading to negatively charged NEs unable to bind DNA in further applications.

## Influence of copolymer amounts on NE stability and size

The size and stability data corresponding to NEs prepared with various amounts of copolymer B are reported in Table 5. A significant decrease in the droplet size when the amount of copolymer is increased is observed. This phenomenon could be explained by the reduction of the interfacial free energy of the dispersed

**Table 5** Influence of amounts of copolymer in the formulation on the stability and the size of the NEs obtained (polydispersity index: $\mu_2/\Gamma^2 < 0.17$ for all measurements)

| | Stability (months) | Size (nm) |
|---|---|---|
| $NE_0$ | | 200 |
| $NEB_9$ | > 9 | 150 |
| $NEB_{25}$ | 6 | 137 |
| $NEB_{50}$ | 1.5 | 120 |

system owing to the presence of the copolymer at the interface as already observed for poly(isobutyl cyanoacrylate) nanocapsules [7].

Surprisingly, the stability on storage was found to decrease with increasing amounts of polymer in the formulation. This observation is in parallel with the data of Fig. 6a, which show that the more polymer used in the formulation, the lower the global charge, and suggests that the colloidal stability of the emulsions should be of an electrostatic nature. As a consequence, the question of the role of the Pluronic in the colloidal stability should be raised at this point. Since the long-term stability is dependent on electrostatic repulsive interactions, it means that the nonionic surfactant is poorly involved in this stabilization process. This is confirmed by our NMR analysis of the ultrafiltrates of the NEs, which revealed the presence in the liquid phase of the vast majority of the Pluronic, proving that the surfactant was not adsorbed at the surface of the nanodroplets, whose interface is not hydrophobic enough to strongly interact.

## Observation of NEs

Despite particle coating by copolymer, the NEs investigated appeared to be fragile colloidal systems (liquid/liquid dispersions). This explains why the observation by classical techniques of microscopy (SEM, TEM) could hardly be applied. SEM (Fig. 7) was found to provide valuable information about the coating of the particles by copolymer. Indeed, differences in the morphologies could be observed when comparing $NE_0$ and $NEB_{25}$. The surface of $NE_0$ appeared smooth, whereas that of $NEB_{25}$ seemed rough and sometimes hairy. These observations strongly suggested the presence of copolymer B on the particle surface. However, this technique seems to be destructive with regards to the sample preparations (aggregation of particles).

The freeze-fracture microscopy technique was also used to examine the NE dispersions and Fig. 8 shows micrographs obtained with $NEB_{25}$. The observations roughly corroborated the size measured by quasielastic light scattering (100–150 nm).

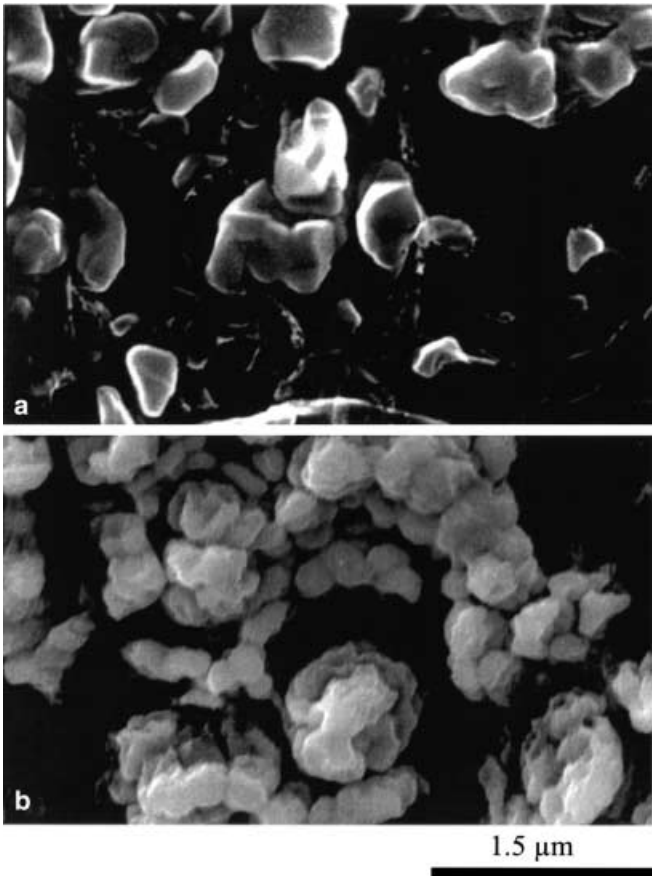


**Fig. 7** Scanning electron micrographs of **a** $NE_0$ and **b** $NEB_{25}$ nanoemulsions

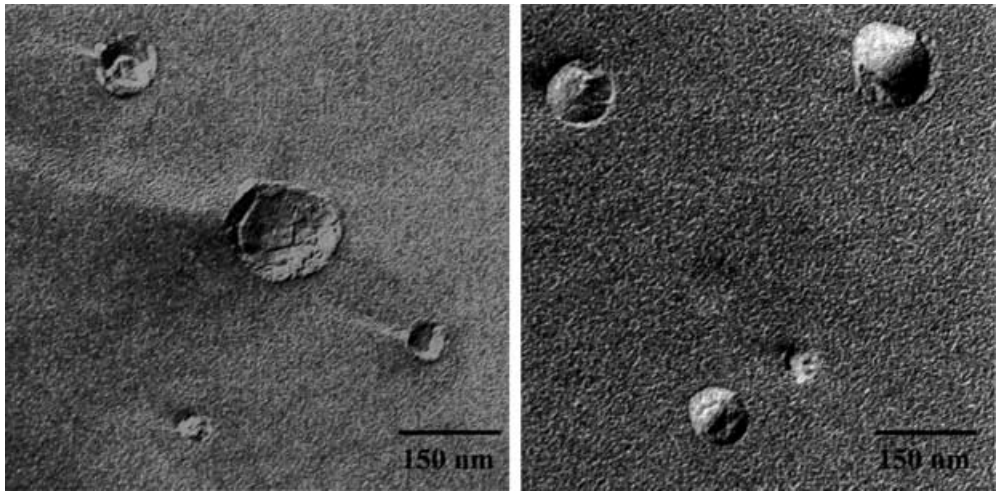


**Fig. 8** Transmission electron micrograph of $NEB_{25}$ nanoemulsion after freeze-fracturing

## Conclusion

With the aim of using NEs as nucleic acid delivery agents, our objective was to functionalize the NE interface so as to be able to envision the surface grafting of a fusogenic peptide, for instance.

Our strategy was based on the interfacial deposition, during NE formation, of a polymer bearing ad hoc functionalities. So the first step of this investigation was to synthesize and to characterize a variety of derivatized polymers. Decylamine was used as a hydrophobic arm to anchor the polymer at the interface of the NE via hydrophobic interactions with the oily core of the droplets. *N*-Acetyl spermine or spermidine were used to produce cationic charges at the interface.

Our investigation showed that in order to obtain stable functionalized NEs the polymer should bear both hydrophobic and cationic arms. Since the modified copolymers are rich in carboxylate moieties, their presence at the interface was detected by modification of the interfacial charge of the NEs. Increasing the amounts of nanodeposited polymer resulted in a reduction in the zero charge point values from 10 (no polymer added) to 5 pH units (saturation of the surface). The presence of a cationic arm allowed the maintenance of a zero charge point value of 6.5 for the functionalized emulsions at saturation.

From a colloidal stability standpoint, the presence of the copolymer allowed the reduction of the mean diameter of the dispersed system, the polymer acting as a surface-active agent during the emulsification process. However, the more the polymer was deposited at the interface, the lower both the global net charge and the long-term stability, suggesting that the colloidal stability was achieved via repulsive electrostatic interactions. This is in accordance with NMR investigation of the ultrafiltrates of the emulsions, which showed that the nonionic surfactant used in the formulation was not strongly adsorbed at the surface of the nanodroplets and so did not confer steric stabilization to the emulsions.

Further studies are in progress to determine the effect of the adsorbed polymer on the adsorption of oligonucleotides onto the polymer functionalized NEs.

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