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

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 acetylpyrmine (or acetylpyrmineid) 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 (Mw: 67,000) [P(MAMVE)] was from Polysciences (Warrington). Decylamine was obtained from Aldrich (Steinheim). N'-acylperasimide dihydrochloride and acetylperasimide 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), lipid 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'-acylperasimide and 45% decylamine with respect to total anhydride functionality) was synthesized as follows. N'-acylperasimide dihydrochloride (100 mg, 0.384 mmol, Mw = 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 µl (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 µl). The polymer was dialyzed against water and lyophilized. The total reaction yield was about 70%.

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

The copolymers obtained were characterized by 1H NMR spectroscopy (Varian 500 MHz) and fluorimetry (Perkin Elmer LSS0) 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 µl was taken from the reaction mixture and added to an excess of fluorescamine (solubilized in 300 µl DMSO). After 30 min of storage in the dark, the sample was diluted 20-fold in DMSO for the fluorimetry analysis (λexc = 420 nm, λem = 477 nm). The fluorescence intensity obtained was related to the concentration of residual amines (N'-acylperasimide dihydrochloride, acetylperasimide trihydrochloride or decylamine) using a calibration curve Ical1 = f([N'-acylperasimide dihydrochloride], [acetylperasimide trihydrochloride] or [decdylamine]) 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 µl), lipid 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 µl trifluoroacetic 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

Ultracentrifugation was performed on NEs (0.1 µm cutoff, Ultratfree MC Millipore, Bedford, USA.). The ultracentrifuged (volume v) was added to a solution of trioxane in D2O (volume v, concentration ci) and analyzed by 1H NMR spectroscopy (200 MHz, Bruker).

The total amount of nonionic surfactant Pluronic F68, HO–(CH2–CH2–O)n–(CH2–CH(CH3)O)2n–(CH2–CH2O)m–H, in the aqueous phase of the NE preparations (volume V) could be estimated by the following equation:

\[ m_p = \left( \frac{c_i \times \frac{I_p}{I}}{695} \times \frac{6}{M_p} \times \frac{V}{v} \right) \]

Ip is the peak integral of the –CH3– groups of the trioxane (δ = 5.2 ppm) (corresponding to six protons), Ip is the peak integral of the –CH2– groups of the Pluronic (δ = 3.4–3.75 ppm) (corresponding to 695 protons) and Mp is the molecular weight of Pluronic (6350 g/mol).

The relative amount of copolymer A in the aqueous phase was determined by comparing the peak integral of the methyl (N'-acetylperasimide dihydrochloride) group of the ultrafiltrate NMR spectrum (Iun) with that detected in the emission spectrum (Iv), 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 –CH2 groups of Pluronic (1.14 ppm) was used as a reference for the spectra of both the emulsion and the ultrafiltrate. The ratio Iun/Iv 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