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Phospholipid–polyaspartamide micelles for pulmonary delivery of corticosteroids

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(DSPE-PEG_{2000}-NH_2)
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ABSTRACT

A novel drug delivery system for beclomethasone dipropionate (BDP) has been constructed through self-assembly of a pegylated phospholipid–polyaminoacid conjugate. This copolymer was obtained by chemical reaction of α,β-poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) with 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)2000] (DSPE-PEG_{2000}-NH_2). Benefiting from the amphiphilic structure with the hydrophilic shell based on both PHEA and PEG and many hydrophobic stearoyl tails, PHEA-PEG_{2000}-DSPE copolymer was able to self assemble into micelles in aqueous media above a concentration of 1.23×10^{-7} M, determined by fluorescence studies. During the self-assembling process in aqueous solution, these structures were able to incorporate BDP, with a drug loading (DL) equal to 3.0 wt%. Once the empty and BDP-loaded micelles were prepared, a deep physicochemical characterization was carried out, including the evaluation of mean size, PDI, ζ potential, morphology and storage stability. Moreover, the excellent biocompatibility of both empty and drug-loaded systems was evaluated either on human bronchial epithelium (16HBE) or on red blood cells. The cellular uptake of BDP, free or blended into PHEA-PEG_{2000}-DSPE micelles, was also evaluated, evidencing a high drug internalization when entrapped into these nanocarriers and demonstrating their potential for delivering hydrophobic drugs in the treatment of pulmonary diseases.

1. Introduction

Nanotechnological approaches have considerable potential for the treatment of pneumological diseases, especially asthma due to the opportunity of polymeric nanocarriers to control the temporal as well as the spatial distribution of drugs within the lung (Bai and Ahsan, 2009; Courrier et al., 2002; Smola et al., 2008; Yang et al., 2010). By varying the composition, structure and size, nanocarriers could offer a controlled and prolonged duration of effect of the encapsulated drugs as well as a regional and cell-specific drug targeting within the lung. In fact, by modulating size of either nanocarriers or dispersion droplets containing these nanostructures, it is possible to determine the deposition zone of these systems into the lung, while their surface decoration with specific cell ligands could also enhance uptake and promote the drug targeting towards a cell type.

In this context, amphiphilic biocompatible copolymer-based nanocarriers have stimulated increasing interest thanks to their great range of applications (Lukyanov and Torchilin, 2004). In particular, being biological membranes formed by lipids and proteins, the synthesis of novel synthetic poliaminoacidic-like structure polymers bearing phospholipids in the side chains has been of great benefit in designing new materials with great potential as drug delivery systems as well as stable coating for nanomaterials (Goodwin et al., 2009; Osada et al., 2009). The use of phospholipid moieties as hydrophobic blocks capping hydrophilic polymer chains can provide additional advantages for particle stability when compared with conventional amphiphilic copolymers due to the existence of two fatty acid acyls in each phospholipids residue, which might contribute considerably to an increase in the hydrophobic interactions between the polymeric chains in the forming micelle core (Torchilin, 2007). Besides the micelle core

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so obtained can offer an efficient cargo space for encapsulation of a variety of sparingly water-soluble therapeutic and diagnostic agents (Lukyanov and Torchilin, 2004).

Some conjugates of phospholipids with water-soluble polymers are commercially available, or can be easily synthesized (Klibanov et al., 1990; Lukyanov and Torchilin, 2004). Poly(ethylenglycol)–phosphatidylethanolamine (PEG–PE) conjugates have been introduced into the area of controlled drug delivery as polymeric surface modifier for liposomes, although each molecule itself represents a characteristic amphiphilic polymer able to form spontaneously micelles with the size of 7–35 nm in an aqueous environment (Dabholkar et al., 2006; Klibanov et al., 1990; Lasic et al., 1991; Wang et al., 2010). However, the use of a multifunctional polymer as starting hydrophilic material to obtain micelles instead of that with a monofunctionality, such as PEG, is a current strategy to improve their efficacy in drug delivery (Ko et al., 2009; Lukyanov and Torchilin, 2004). In this direction, amphiphilic poly(vinylpyrrolidone) (PVP)–lipid conjugates with various polymer lengths have been prepared for the solubilization of poorly water-soluble drugs yielding highly stable biocompatible formulations (Lukyanov and Torchilin, 2004).

We have recently focused our attention on the realization of amphiphilic polymeric derivatives based on α,β-poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) able to form micelle-like aggregates with great potential as delivery systems for poorly water soluble drugs (Cavallaro et al., 2003, 2004; Craparo et al., 2008, 2009).

In this work, we report the synthesis of a pegylated phospholipid–polyaminoacid conjugate able to form core–shell aggregates. In this conjugate, the chosen phospholipid derivative, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)2000] (DSPE-PEG_{2000}-NH_2), was chemically bound to the hydroxyl pendant groups of α,β-poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) in a such way to obtain both a multifunctional and a pegylated polymeric derivative (PHEA-PEG_{2000}-DSPE copolymer). In particular, the grafting reaction of DSPE-PEG_{2000}-NH_2 on PHEA backbone was carried out by using N,N′-disuccinimidyl carbonate (DSC) as activating agent of PHEA hydroxyl pendant groups. The obtained copolymer, properly characterized in terms of FT-IR, ^{1}H NMR and SEC analyses, was found to be able to form easily self-assembled systems in water at low concentrations. In particular, the formation of core–shell type micelles was confirmed by fluorescence studies, and then the obtained micelles were characterized in terms of dimensional analysis, ζ potential values and morphology.

On the basis of the well-known capability of nanocarriers such as polymeric micelles to solubilize an adequate amount of hydrophobic drugs, to pass through the mucus layer associated with bronchial inflammatory diseases escaping from pulmonary phagocytosis due to their bulky hydrophilic outer shell, the potential of PHEA-PEG_{2000}-DSPE micelles as pulmonary delivery systems for a corticosteroid such as beclomethasone dipropionate (BDP) was investigated (Gaber et al., 2006; Marsh et al., 2003; Smola et al., 2008). In fact, the efficacy of this kind of drug in the treatment of bronchial inflammatory diseases fails due to the inability of such drugs to penetrate through the mucus layer to reach the target site (Smola et al., 2008). Moreover, the presence of enhanced level of a mammalian secreted phospholipase A2 in different pathological sites, that is able to degrade pegylated phosphatidyl ethanolamine residues, could increase the drug release into the lung (Davidsen et al., 2001; Gaber et al., 2006; Vermehren et al., 2001). To investigate the effects on cell viability of both empty and drug-loaded micelles and the capability of BDP entrapped into these structures to penetrate into human bronchial epithelial (16 HBE) cells, in vitro tests were carried out.

2. Materials and methods

2.1. Chemicals

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)$_{2000}$] (DSPE-PEG_{2000}-NH_2) was purchased from Avanti Polar Lipids, Inc. Anhydrous *N,N*-dimethylacetamide (DMA), disuccinimidyl carbonate (DSC), CH_2Cl_2, acetone, DMF-d_7 (isotopic purity 99.9%), and pyrene were purchased from Sigma–Aldrich (Italy). Diethyl ether was purchased from Fluka (Italy).

α,β-Poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) was prepared and purified according to a procedure reported elsewhere (Craparo et al., 2006; Giammona et al., 1987). Spectroscopic data (FT-IR and ^{1}H NMR) were in agreement with previous results (Mendichi et al., 2000). PHEA weight-average molecular weight ($\bar{M}_w$), determined by size exclusion chromatography (SEC) analysis, was found to be 41.4 kDa ($\bar{M}_w/\bar{M}_n = 1.8$).

SEC system (Waters, Mildford, MA) was equipped with a pump system, two Phenogel columns from Phenomenex (5 μm particle size, 10^3 Å and 10^4 Å of pores size) and a 410 differential refractometer (DRI) as concentration detector, all from Waters (Mildford, MA). The $\bar{M}_w$ values of PHEA and PHEA-PEG_{2000}-DSPE were determined by using PEO/PEG as standards (range 232–932000 Da), DMF + 0.01 M LiBr as mobile phase, a flow of 0.8 mL min^{-1}, operating at 50 °C (±0.1 °C).

2.2. PHEA-PEG_{2000}-DSPE graft copolymer synthesis

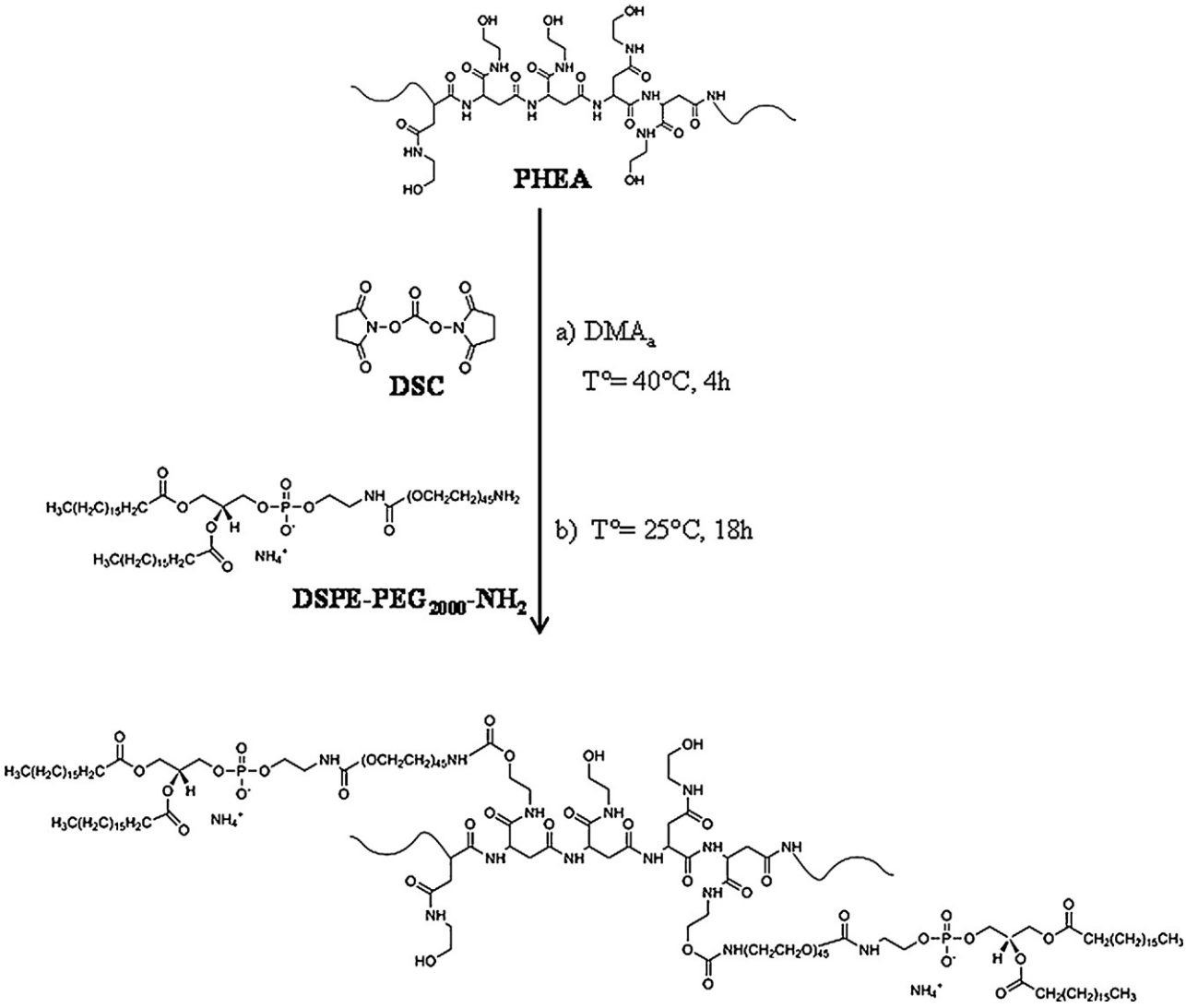
To an organic solution of PHEA (40 mg mL^{-1}) in anhydrous DMA, a proper amount of DSC was added in such way to have R_1 = moles of DSC/moles of PHEA repeating units equal to 0.08. The reaction mixture was kept at 40 °C for 3.5 h and then added to a solution of DSPE-PEG_{2000}-NH_2 in anhydrous DMA (5.8 mg mL^{-1}) in a such way to have R_2 = moles of DSPE-PEG_{2000}-NH_2/moles of PHEA repeating units equal to 0.025. The obtained mixture reaction was left at 25 °C for 18 h under argon and continuous stirring. After this time, the reaction solution was added drop wise to 100 mL of diethyl ether; the obtained precipitate was washed four times in a mixture of diethyl ether/CH_2Cl_2 (2:1,v/v) and then one time in acetone. The synthetic procedure and the chemical structure of PHEA-PEG_{2000}-DSPE graft copolymer have been depicted in Scheme 1.

PHEA-PEG_{2000}-DSPE graft copolymer, obtained as dried product, was dissolved in twice-distilled water and the obtained colloidal dispersion was dried by freeze-drying. The product was obtained with a yield of 110 wt% based on the starting PHEA.

2.3. Critical aggregation concentration (CAC) determination by fluorescence spectroscopy

A stock solution of pyrene (6.0×10^{-2} M) was prepared in acetone and stored at 5 °C. To obtain the excitation spectrum, the pyrene solution was diluted with bi-distilled water to a pyrene concentration of 12×10^{-7} M. The solution was distilled under vacuum at 60 °C for 2 h to remove acetone. Then the acetone-free pyrene solution was mixed with PHEA-PEG_{2000}-DSPE graft copolymer aqueous dispersions, which ranged in concentration from 1×10^{-5} g L^{-1} to 1.5 g L^{-1}. The final concentration of pyrene in each sample was 6.0×10^{-7} M. Solutions were placed in quartz cuvettes and out-gassed by bubbling with oxygen-free nitrogen for 5 min before recording spectra.

Excitation spectra were recorded on RF-5301PC spectrofluorometer (Shimadzu, Italy). The slit openings were 3 mm. The change



Scheme 1. Synthetic procedure of PHEA-PEG$_{2000}$-DSPE graft copolymer.

of the intensity ratio (I_{337}/I_{333}) of the pyrene as a function of copolymer concentration was plotted from excitation spectra from 300 to 360 at emission wavelength 390 nm (Gao et al., 2008).

2.4. Micelle characterization: mean size and ζ potential

The mean diameter and width of distribution (polydispersity index, PDI) of each sample were determined by photon correlation spectroscopy (PCS) by using a Zetasizer Nano ZS (Malvern Instrument, Malvern, UK). The measurements were carried out at a fixed angle of 90° and at the temperature of 25 °C on each dispersion (5 mg mL^{-1}) obtained by using bi-distilled water, NaCl 0.9 wt% aqueous solution and phosphate buffered saline solution (PBS) at pH 7.4 as suspending media. Each dispersion was kept in a cuvette and analyzed in triplicate. The deconvolution of the measured correlation curve to an intensity size distribution was accomplished by using a non-negative least squares algorithm.

2.5. Preparation of BDP-loaded PHEA-PEG$_{2000}$-DSPE micelles

The preparation of BDP-loaded micelles was in according with a published procedure (Cavallaro et al., 2003; Craparo et al., 2009). In particular, BDP-loaded systems were prepared by closely mixing, using pestle and mortar, an appropriate amount of PHEA-PEG$_{2000}$-DSPE and BDP to obtain a final copolymer/drug weight ratio equal to 4:1. Then, 3 mL of ethanol was added and, then, after complete evaporation of the solvent, aliquots of 500 μL of water were added until reaching 10 mL of volume. The obtained dispersion was submitted to centrifugation at 11,800 rpm, at 25 °C for 10 min, filtration on cellulose acetate filters (0.2 μm) and lyophilisation.

2.6. Drug loading (DL%) determination

To determine BDP amount blended into PHEA-PEG$_{2000}$-DSPE micelles and drug release profiles, an HPLC method able to disaggregate the drug-loaded micelles was developed. In particular, the HPLC analysis was performed using a C18 column (μBondpack, 5 μm, 250 mm × 46 mm i.d., obtained from Waters); mobile phase was a mixture methanol:water 30:70 (v/v) with a flow rate of 1 mL min^{-1}, reading at λ 238 nm. In order to determine the drug loading (DL%), 5 mg of BDP-loaded systems was dissolved in 5 mL of bi-distilled water, filtered with cellulose membrane filters (0.2 μm) and analyzed by HPLC. The obtained peak area corresponding to BDP amount blended into PHEA-PEG$_{2000}$-DSPE micelles was compared with a calibration curve obtained by plotting areas versus standard solution concentrations of BDP in methanol in the range of 100–10 μg mL^{-1} ($y = 43401 \times 10^{-3}x$, $R^2 = 0.99696$). Results were expressed as the

weight percent ratio between the loaded BDP and the dried system (micelles + BDP).

In order to ensure that the drug is not absorbed within the cellulose filter, several BDP solutions at known concentrations were filtered and the concentration values, before and after filtration, were evaluated by HPLC analysis. No significant differences in drug concentrations were evidenced.

2.7. Solubility studies

BDP, free of loaded into micelles water solubility was determined by shaking an excess solute in water at 25 °C. The suspension was sonicated for 10 min, centrifuged at 8000 rpm for 10 min, filtered on 0.45 mm cellulose membrane. BDP amount in saturated solution was evaluated by HPLC analysis as described above.

BDP water solubility was also evaluated on drug-loaded micelles as follows: increasing amounts ranging from 10 to 500 mg of dried BDP-loaded micelles were dispersed in 5 mL of water. The obtained dispersions were filtered on 0.45 mm cellulose membrane and analyzed by HPLC.

2.8. Field emission scanning electron microscopy (FE-SEM)

The nano-morphology of the deposited micelle sample was investigated by using a high brilliance LEO 1530 (FE-SEM) apparatus equipped with an energy dispersive X-ray spectrometer (EDS) model INCA 450 and a back scattered electron detector. Prior to microscopic examination, the aqueous dispersion of PHEA-PEG_{2000}-DSPE micelles at a concentration of 0.25 mg/mL was drop-cast onto Si surface and dried by overnight evaporation of water at room temperature.

2.9. Storage stability

Both lyophilised empty and or drug-loaded PHEA-PEG_{2000}-DSPE micelles were stored at 0 °C for 3 months in the dark. After this time, samples were dispersed in bi-distilled water and characterized in terms of mean size, PDI and ζ potential. Moreover, chemical stability of loaded BDP was evaluated by HPLC analysis, as above reported.

2.10. Stability studies in PBS/ethanol

A mixture between an isotonic PBS at pH 7.4 and ethanol 80:20 (v/v) was used to obtain a release profile of BDP from PHEA-PEG_{2000}-DSPE micelles. In particular, 10 mg of dried BDP-loaded PHEA-PEG_{2000}-DSPE micelles was suspended in the mixture and transferred inside of a Spectra/Por dialysis membrane (MWCO 14 Da). This dialysis membrane was immersed into 20 mL of pre-heated release medium and incubated at 37 ± 0.1 °C under continuous stirring in a Benchtop 80 °C Incubator Orbital Shaker model 420.

At scheduled time intervals, solution aliquots (1 mL) were taken out from the outside of the dialysis membrane and replaced with fresh PBS/ethanol mixture. The drawn samples were filtered by cellulose filters (0.2 μm) and analyzed by HPLC in order to determine the released BDP amount. Profile releases were determined by comparing the amount of released drug as a function of incubation time with the total amount of drug loaded into the micelles. Data were correct taking in account the dilution procedure.

A control experiment to determine the release behaviour of the free drug was also performed: an appropriate amount of BDP was dispersed in the PBS at pH 7.4 and ethanol 80:20 (v/v) mixture, in order to have a BDP final concentration equal to whom of BDP-loaded PHEA-PEG_{2000}-DSPE micelles, put into a dialysis tube (MWCO 14 kDa) and immersed into the proper medium. The amount of BDP was detected as reported above.

2.11. Cell viability assay on human bronchial epithelial cells (16HBE)

Cell viability was assessed by the MTS assay on 16HBE cell line (obtained as a gift from the Istituto di Biomedicina e Immunologia Molecolare, Consiglio Nazionale delle Ricerche, Palermo, Italy), at pH 7.4, using a commercially available kit (Cell Titer 96 Aqueous One Solution Cell Proliferation assay, Promega) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate. 16HBE cells were maintained in minimum essential medium (MEM) containing 10 vol% foetal calf serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B (Sigma–Aldrich, Italy) under standardized conditions (95% relative humidity, 5% CO_2, 37 °C).

16HBE cells were plated at a density of 1×10^5 cells mL^{-1} on a 96-well plate. Upon reaching confluence, 16HBE cells were incubated with 10 μL per well of cell culture medium containing micelle-entrapped BDP at a final concentration per well ranging between 7×10^{-4} and 7×10^{-7} M. Moreover, cell viability was also carried out by incubating cells in the presence of empty micelles, at concentrations equal to those used for drug loaded-micelles. After 24 and 48 h of incubation, the growth medium was replaced with 100 μL of fresh MEM, 20 μL of a MTS/PMS solution was added to each well and plates were returned to incubate for an additional 3 h at 37 °C. The absorbance at 490 nm was read using a Microplate reader (Multiskan Ex, Thermo Labsystems, Finlandia). Relative cell viability (percentage) was expressed as (Abs_{490} treated cells/Abs_{490} control cells) × 100, on the basis of three experiments conducted in multiple of six. Cells incubated with the medium were used as negative control.

2.12. Haemolysis test

Human erythrocytes were obtained from voluntary healthy blood donors. Human erythrocytes isolated from fresh citrated-treated blood were collected by centrifugation at 2200 rpm for 10 min at 4 °C. The pellet was washed four times with PBS at pH 7.4 by centrifugation and suspended in the same buffer. The erythrocyte pellet was diluted in PBS at pH 7.4 to a final concentration of 4% erythrocytes. This stock dispersion was always freshly prepared and used within 24 h after preparation. Empty or drug-loaded PHEA-PEG_{2000}-DSPE micelle dispersions in PBS were added to the erythrocyte suspension and incubated for 1 h at 37 °C under constant shaking (final copolymer concentrations = 0.5, 0.05 and 0.005 mg mL^{-1}). After centrifugation, the release of haemoglobin was determined by photometric analysis of the supernatant at 540 nm. Complete haemolysis was achieved by using an aqueous solution of Triton X-100 (5 wt%) (100% control value) while 0% lysis by using PBS at pH 7.4 (blank value). Each experiment was performed in triplicate and repeated twice. The erythrocyte lysis percentage was calculated according to the following formula:

$$\%\,\text{lysis} = \frac{A_{\text{micelles}} - A_{\text{blank}}}{A_{100\,\%\,\text{lysis}} - A_{\text{blank}}} \times 100$$

where A_{micelles} is the absorbance value of the haemoglobin released from erythrocytes treated with micelle dispersion, A_{blank} is the absorbance value of the haemoglobin released from erythrocytes treated with PBS buffer, and $A_{100\%\,\text{lysis}}$ is the absorbance value of the haemoglobin released from erythrocytes treated with 5 vol% Triton X-100 solution.

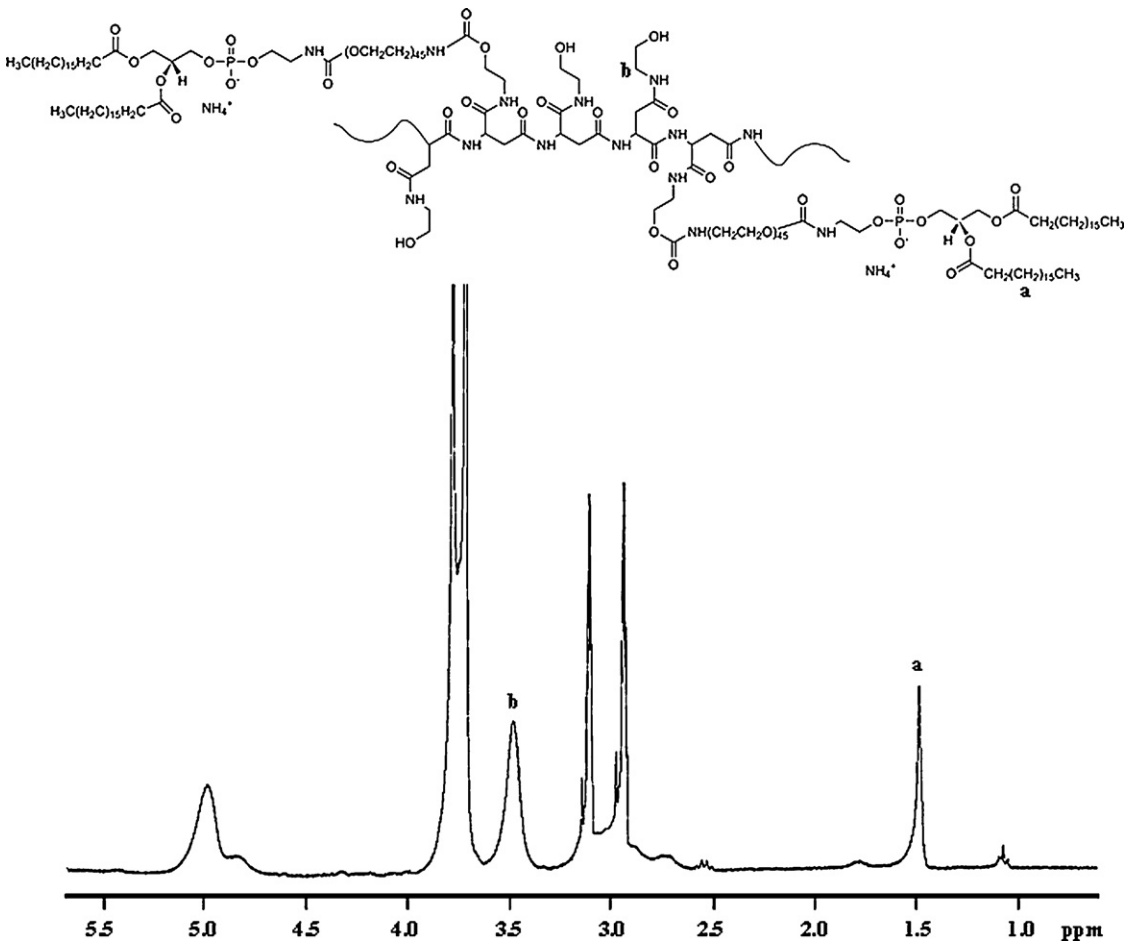


Fig. 1. ^{1}H NMR spectrum of PHEA-PEG$_{2000}$-DSPE graft copolymer in DMF-d_7.

2.13. Cell drug uptake studies

To analyze drug and drug-loaded copolymer micelle uptake, 16HBE cells (2×10^5 cells mL^{-1}) maintained in normal medium were cultured in a 24-well plate at 37 °C in an atmosphere of 5% CO_2 for 1–2 h until they formed a confluent monolayer. Upon reaching confluence, the culture medium was removed, cells were washed twice with 0.5 mL of Dulbecco's modified Eagle's medium (DPBS, Sigma) and then 0.5 mL of fresh MEM was added.

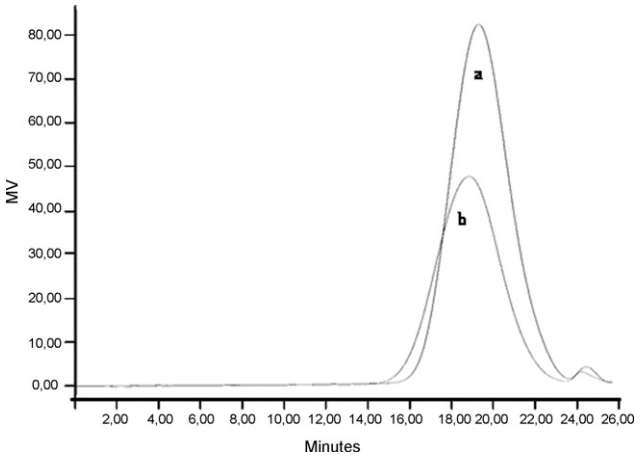


Fig. 2. SEC chromatograms of (a) PHEA and (b) PHEA-PEG$_{2000}$-DSPE copolymers.

After pre-incubation at 37 °C for 30 min, aliquots of micelle-encapsulated drug or drug suspension were added to each well in order to obtain a final drug concentration equal to 7×10^{-5} M and incubated for 24 and 48 h. Following the incubation period, the medium was removed. The cell monolayer was washed twice with DPBS and the washings were combined with the incubation medium. The cell monolayer was then treated with appropriate volume of CelLytic MT reagent (Sigma–Aldrich, Italy). After incubation for 15 min on a shaker, cells were scraped and the cell lysate collected and lyophilised. The amount of drug present in the cell lysate and in the washing media was quantified by HPLC. It is noteworthy that the sum of internalized and not-internalized drug was always about 100% (data not reported).

2.14. Statistical analysis

The statistical analysis of the samples was performed by using a Student's *t*-test and *p*-values <0.05 were considered statistically significant. All data were reported as mean ± S.D., unless otherwise stated.

3. Results and discussion

3.1. Synthesis and characterization of PHEA-PEG$_{2000}$-DSPE graft copolymer

In this paper, the phospholipid derivative 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)$_{2000}$] (DSPE-PEG$_{2000}$-NH$_2$) was chemically grafted to the hydroxyl

groups of α,β-poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) to obtain the PHEA-PEG_{2000}-DSPE graft copolymer. In this way, both a multifunctional and a pegylated polymeric derivative was obtained thanks to the presence PHEA and PEG, respectively. Moreover, the grafting of each phospholipid residue permits to introduce two hydrophobic stearoyl tails that could considerably contribute to obtain very stable micelles due to extensive hydrophobic interactions among long chains attached to the forming micelle core (Torchilin, 2007).

The derivatization reaction was carried out by a single synthetic step in organic solvent involving the activation of PHEA hydroxyl groups via N,N′-disuccinimidyl carbonate (DSC) for 4 h at 40 °C and then the coupling reaction with DSPE-PEG_{2000}-NH_2 for 18 h at 25 °C (see Section 2).

The amount of DSC and DSPE-PEG_{2000}-NH_2 was determined according to $R_1 = 0.08$ and $R_2 = 0.025$, being:

$$R_1 = \frac{\text{moles of DSC}}{\text{moles of repeating units of PHEA}}$$

and

$$R_2 = \frac{\text{moles of DSPE-PEG}_{2000}\text{-NH}_2}{\text{moles of repeating units of PHEA}}$$

The obtained product was insoluble in organic solvents, such as dichloromethane and acetone, while is soluble in dimethylsulfoxide and N,N′-dimethylformamide, and easily dispersible in water.

The ^{1}H NMR spectrum of PHEA-PEG_{2000}-DSPE graft copolymer has been reported in Fig. 1.

^{1}H NMR spectrum in DMF-d_7 showed: δ 1.08 (1d, $6H_{DSPE\text{-}PEG}$ $\mathbf{H_3C}(CH_2)_{14}CH_2$–); δ 1.48 (*m*, $60H_{DSPE\text{-}PEG}$ $H_3C(C\mathbf{H}_2)_{15}CH_2$–); δ 1.79 (*m*, $2H_{DSPE\text{-}PEG}$ $H_3C(CH_2)_{15}C\mathbf{H}_2$–); δ 3.01 (*m*, $2H_{PHEA}$, –$CH\mathbf{CH_2}C(O)NH$–); δ 3.48 (*t*, $2H_{PHEA}$ –$NH\mathbf{CH_2}CH_2O$–); δ 3.74 (*t*, $2H_{PHEA}$ –$NHCH_2C\mathbf{H}_2O$–); δ 3.78 (*t*, 178 $H_{DSPE\text{-}PEG}$ –$CH_2\mathbf{CH_2}O$–) and δ 4.98 (*m*, $1H_{PHEA}$ –$NH\mathbf{CH}C(O)CH_2$–).

The degree of derivatization in -PEG_{2000}-DSPE residues ($DD_{DSPE\text{-}PEG}$) for PHEA-PEG_{2000}-DSPE graft copolymer was calculated by comparing the integral of the peak related to protons at δ 1.48 awardable to $H_3C(\mathbf{CH_2})_{15}CH_2$– belonging to linked -$PEG_{2000}$-DSPE residues (signals **a** in the ^{1}H NMR spectrum of Fig. 1) with the integral related to protons at δ 3.48 awardable to –$NH\mathbf{CH_2}CH_2O$– belonging to PHEA (signal **b** in the ^{1}H NMR spectrum of Fig. 1). The $DD_{DSPE\text{-}PEG}$ was expressed as mean value of three determinations and resulted to be 1.5 ± 0.3 mol%. In other words, this value means that three stearoyl tails are inserted on the PHEA backbone every one hundred repeating units. The presence of a negligible amount of unreacted DSPE-PEG_{2000}-NH_2 (0.13 mol% on the total amount linked to the PHEA backbone) in the purified copolymer was determined by HPLC analysis (data not shown).

In order to verify the occurrence of PHEA derivatization reaction with DSPE-PEG_{2000}-NH_2, the weight-average molecular weight ($\bar{M}_w$) and the polydispersity index ($\bar{M}_w/\bar{M}_n$) values of both starting PHEA and obtained PHEA-PEG_{2000}-DSPE graft copolymer were obtained in organic environment by SEC analysis (see Section 2).

In Fig. 2, the overlapped SEC chromatograms of PHEA-PEG_{2000}-DSPE and PHEA graft copolymers are reported.

It was found that the $\bar{M}_w$ value for PHEA was equal to 41.4 kDa ($\bar{M}_w/\bar{M}_n = 1.8$) while for PHEA-$PEG_{2000}$-DSPE graft copolymer it was found to be equal to 61.0 kDa ($\bar{M}_w/\bar{M}_n = 1.9$). The latter is in accordance with the theoretical one calculated considering the initial PHEA $\bar{M}_w$ and the resulting $DD_{DSPE\text{-}PEG}$ value. Therefore, it can be also considered that no degradation phenomena occurred in the PHEA backbone due to the reaction conditions chosen for obtaining the PHEA-PEG_{2000}-DSPE graft copolymer.

The FT-IR spectrum of PHEA-PEG_{2000}-DSPE graft copolymer is reported in Fig. 3. It showed a broad band centered at 3400 cm^{-1} (asymmetric stretching of νOH and νNH_{PHEA}); bands at 2917 cm^{-1} (stretching νCH_{PHEA} and $\nu CH_{DSPE\text{-}PEG}$); 1656 cm^{-1} (νCO amide I_{PHEA}); 1543 cm^{-1} (νCO amide II_{PHEA}) and 1110 cm^{-1} (stretching $\nu C\text{–}O_{DSPE\text{-}PEG}$). The comparison between this spectrum with DSPE-PEG_{2000}-NH_2 and PHEA FT-IR spectra also confirmed that the derivatization reactions occurred.

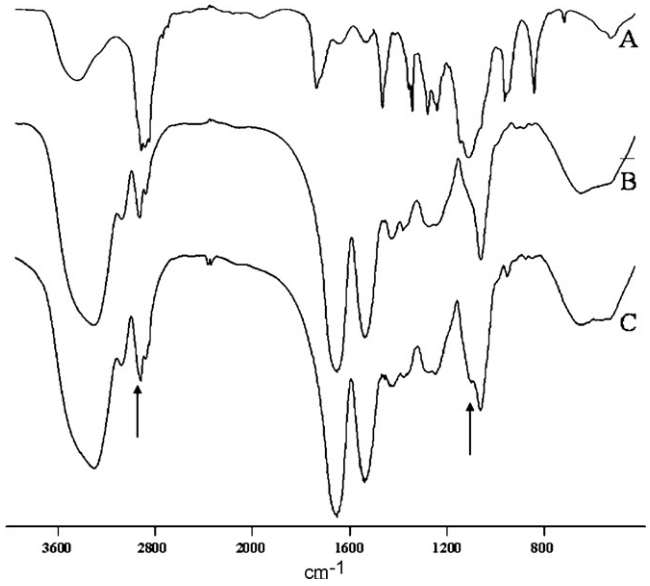


Fig. 3. FT-IR spectra of DSPE-PEG_{2000}-NH_2 (A), PHEA (B) and PHEA-PEG_{2000}-DSPE (C) copolymers. Spectra are recorded in transmittance scale (%*T*).

In fact, it showed the typical peaks of DSPE-PEG_{2000}-NH_2, such as the peak at 1108 cm^{-1} attributable to the C–O stretching of PEG moieties. Moreover, an increased intensity of the peak at 2920 cm^{-1} can be observed, thanks to the contribution of the $–CH_2–$ asymmetric stretching of DSPE-PEG_{2000}-NH_2.

3.2. Preparation and characterization of empty and drug-loaded PHEA-PEG_{2000}-DSPE micelles

Considering the amphiphilic potential of PHEA-PEG_{2000}-DSPE graft copolymer, its tendency towards the self-assembling behaviour after aqueous dispersion was investigated. In fact, the grafting of both hydrophilic and hydrophobic moieties such as PEG and stearoyl residues on the PHEA backbone, performed in this copolymer in one single step, suggested the possibility to promote, in aqueous phase, the polymer arrangement into micelles because of multiple hydrophobic interaction sites (Cavallaro et al., 2004; Lukyanov and Torchilin, 2004).

For this purpose, the critical aggregation concentration (CAC) in water of PHEA-PEG_{2000}-DSPE graft copolymer was determined by fluorescence studies using pyrene as hydrophobic probe (Rosen, 1989). Pyrene would be preferentially allocated into hydrophobic cores of micelles with a change of its photo-physical properties (Gao et al., 2008). The CAC can be obtained by plotting of I_{337}/I_{333} ratios obtained by the excitation spectra of pyrene versus the logarithm of the copolymer aqueous concentrations (Francis et al., 2004). A typical plot of I_{337}/I_{333} versus log *C* (g L^{-1}) for PHEA-PEG_{2000}-DSPE graft copolymer has been shown in Fig. 4.

As can be seen, below a certain concentration, I_{337}/I_{333} ratio values remain uncharged, but with further increasing of copolymer concentrations, this ratio increases significantly until it reaches a plateau, implying the beginning of the self-assembling process. The CAC value was determined by intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentrations (Gao et al., 2008). From this plot, CAC

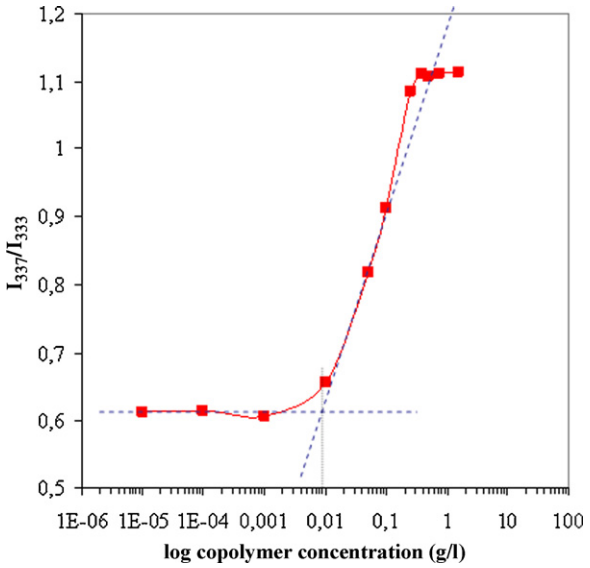


Fig. 4. Plot of the intensity I_{337}/I_{333} ratio (from pyrene excitation spectra) as a function of the PHEA-PEG$_{2000}$-DSPE graft copolymer concentration (1×10^{-5}–1.5 g L^{-1}) for λ_{em} = 390 nm. Each value is the mean of three independent experiments.

value of 7.5×10^{-3} g L^{-1} was determined. This value corresponds to 1.23×10^{-7} M, which is about 1000-fold and 100-fold lower than those of conventional low molecular weight surfactants and PEG–phosphatidylethanolamine (PEG–PE) conjugates, respectively (Lukyanov and Torchilin, 2004). Such low CMC value indicates that micelles prepared from PHEA-PEG$_{2000}$-DSPE graft copolymer will potentially maintain their integrity even upon dilution, for example, in biological fluids such as mucus due to a therapeutic pulmonary application.

The schematic representation of the core–shell structure formation starting from PHEA-PEG$_{2000}$-DSPE graft copolymer in aqueous dispersion is depicted in Fig. 5.

To confirm the formation of colloidal nanostructures, these micelles were also characterized in terms of mean size and PDI values in three different dispersing aqueous media by using photon correlation spectroscopy (PCS), and the analytical data are reported in Table 1.

As can be seen, these nanostructures have a size distribution with an average diameter of 26.4 nm in bi-distilled water, and slightly higher diameters in the other investigated media; these small differences could be attributed to their different ionic

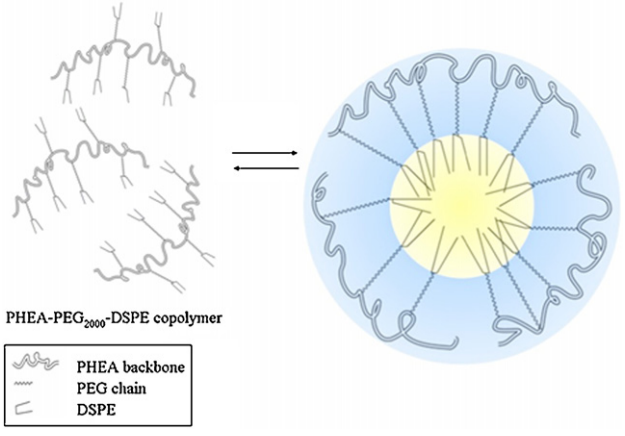


Fig. 5. The schematic representation of the core–shell structure formation starting from PHEA-PEG$_{2000}$-DSPE graft copolymer in aqueous dispersion.

strengths of the media. The surface ζ potential value of these structures, also reported in Table 1, was about −19.03 mV in bi-distilled water and decreases when it is determined in PBS and NaCl aqueous solution, for the potential screening effect of solution ions.

To evaluate the potential of these nanostructures as pulmonary delivery systems for poorly soluble therapeutic agents, beclomethasone dipropionate (BDP), a water insoluble glucocorticoid, was chosen as model drug (Wilcox and Avery, 1973; Umland et al., 2002). Several formulations have been developed to deliver BDP to the pulmonary system including nebulizers, breath-actuated dry powder inhalers (DPI) and metered dose inhalers (MDI). However, the administration of BDP as drug particle suspension via nebulizer rather than hand-held inhalers may have certain advantages, as many patients, such as young children, the elderly or the acutely ills, fail to use these devices properly or efficiently. The entrapment into a vesicular nanostructure may represent an appropriate method for BDP solubilization towards a sustained pulmonary delivery and, at the same time, for a more efficient drug internalization into the pulmonary epithelium because of the capability of nanosized systems to penetrate into cells, i.e. by endocytosis.

To entrap BDP into these micelles, a simple method of drug incorporation, already reported, was chosen (Cavallaro et al., 2004; Craparo et al., 2009). Briefly, this technique involved the simple dispersing of the dry copolymer and the drug in a volatile organic solvent using pestle and mortar, then the evaporation of organic solvent to form a PHEA-PEG$_{2000}$-DSPE/BDP drug film. This latter was then dispersed in bi-distilled water and the amount of drug exceeded the solubilization capacity of micelles was removed by centrifugation and filtration.

To quantify the amount of BDP loaded in PHEA-PEG$_{2000}$-DSPE micelles, an HPLC analysis was carried out (see Section 2). The drug loading (DL%), expressed as weight percentage ratio between the loaded drug and the dried system (micelles + BDP), was 3.0 wt%.

In order to evaluate whether any increase of drug water solubility occurs in the presence of polymeric micelles, solubility studies on BDP alone and on freeze-dried BDP-loaded PHEA-PEG$_{2000}$-DSPE micelles were performed. Under the used experimental conditions, an increase of drug solubility of 240-fold was found in comparison with free drug under the same conditions. In particular, the solubility value increases from 5 μg mL^{-1} to 1.2 mg mL^{-1}.

As for empty systems, also the BDP-loaded micelles were characterized in terms of mean size, polydispersity index (PDI) and ζ potential, and results are reported in Table 1. As can be seen, an increase of mean size was observed for drug-loaded micelles compared to the empty ones, probably for the presence of the drug into the micelle core; BDP also affects the ζ potential values of these systems that slightly decreased in the presence of the loaded drug in all the investigated media.

In order to confirm the nanometer size and to investigate the morphology of both empty and drug-loaded PHEA-PEG$_{2000}$-DSPE micelles (sample freshly prepared and evaporated overnight), FE-SEM was used and obtained images for empty micelles have been reported in Fig. 6.

These images were consistent with findings obtained from dimensional analysis and also revealed a spherical shape of investigated samples. Data coherent with DLS size measurements were obtained from drug-loaded PHEA-PEG$_{2000}$-DSPE micelles (data not shown).

To support their potential as drug delivery systems, the stability of both empty and BDP-loaded PHEA-PEG$_{2000}$-DSPE micelles after storage was evaluated in terms of size, PDI and ζ potential, following a procedure reported elsewhere (Gaber et al., 2006).

In particular, to evaluate micelle stability during storing, either dried empty or BDP-loaded PHEA-PEG$_{2000}$-DSPE micelles were

Table 1
Mean size, PDI and ζ potential values in bi-distilled water, PBS and NaCl 0.9 wt% of empty and drug BDP-loaded PHEA-PEG_{2000}-DSPE micelles.

Sample	Dispersing medium	Mean size (nm)	PDI	ζ potential (mV) (±S.D.)
Empty	H_2O	26.4	0.3	−19.0 (±6.8)
	PBS	31.8	0.4	−7.7 (±2.0)
	NaCl 0.9%	28.7	0.3	−5.7 (±1.1)
BDP-loaded	H_2O	69.2	0.4	−12.3 (±3.1)
	PBS	58.6	0.4	−2.5 (±1.8)
	NaCl 0.9%	66.4	0.4	−3.5 (±2.4)

stored for 3 months at 0 °C. After this time, micelles were dispersed in bi-distilled water and analyzed in terms of mean size, PDI and ζ potential. The obtained results suggested that either empty or BDP-loaded PHEA-PEG_{2000}-DSPE micelles were stable during storing in the used experimental conditions, being size, PDI and ζ potential values comparable to that reported in Table 1 (data not shown). HPLC analysis also suggested that the drug was stable under these storage conditions (data not shown).

To evaluate the ability of these micelles to retain the encapsulated drug under sink conditions and to release it slowly in physiological media, a stability study was carried out in phosphate buffer solution (PBS) at pH 7.4/ethanol mixture (80:20) by evaluating the amount of released drug from micelles at prefixed time intervals across a dialysis tube. The use of this modified dissolution medium (containing ethanol) to test preparation containing poorly aqueous-soluble active substances was in accordance to the European Pharmacopoea (Helle et al., 2010; Pitarresi et al., 2007). Moreover, the BDP diffusion profile alone was investigated in order to determine the diffusion rate of the free drug across the dialysis membrane. The amount of released BDP was expressed as percentage ratio between the weight of released drug at the prefixed time and the total amount of BDP loaded into micelles. In Fig. 7, the drug dissolution and release profile from PHEA-PEG_{2000}-DSPE micelles were reported until 48 h incubation.

Results shown in Fig. 7 clearly indicate that the investigated carrier possesses a great stability, being able to retain more than 70% of initially entrapped drug even after 48 h incubation and could in this way improve the drug internalization into cells, allowing its entry in the micellar form.

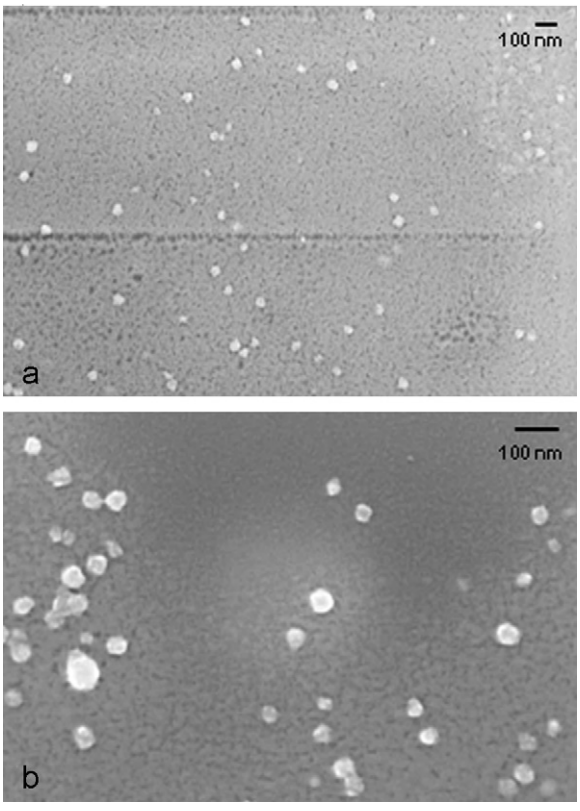


Fig. 6. Representative FE-SEM images of empty PHEA-PEG_{2000}-DSPE micelles. The bars represent 100 nm.

3.3. In vitro experiments

Taking also into account the possibility to incorporate into aerosol droplets the BDP-loaded PHEA-PEG_{2000}-DSPE micelles and to administer them by the pulmonary route, in vitro viability studies were conducted by using the human bronchial epithelial cell line (16HBE) as model epithelial cells.

In effect almost in principle it is possible to use these nanosystems in pulmonary route to maximize local effects into the lung as well as to minimize systemic effects compared to other administration routes, to reduce either the frequency of application or mild cough and/or wheezing due to chemical irritation caused by free drug administered in inhalation dosage form.

Cytotoxicity of BDP-loaded micelles on 16 HBE cells was evaluated by the MTS assay at different drug concentrations (7×10^{-7}–7×10^{-4} M), after 24 and 48 h incubation. Moreover, cell viability was also evaluated in the presence of empty micelles, at concentrations equal to those used for drug-loaded micelles. Results are reported in Fig. 8.

As can be seen, both empty and BDP-loaded PHEA-PEG_{2000}-DSPE micelles showed low cytotoxicity at concentrations ranging between 7×10^{-5} and 7×10^{-7} M, after 24 and 48 h incubation. At the higher tested drug concentration (7×10^{-4} M), these values are lower that 80% after 48 h incubation; this was most likely due to the high copolymer concentration used for solubilizing the drug, as demonstrated by the similar cell viability value obtained by using both empty and drug-loaded PHEA-PEG_{2000}-DSPE micelles.

The excellent biocompatibility of these systems was also confirmed by a haemocompatibility test. Erythrocytes were incubated with aqueous dispersions of both empty and BDP-loaded systems at final copolymer concentrations equal to 0.5, 0.05 and 0.005 mg mL^{-1} for 1 h. Under these conditions, no haemolytic effects were showed, indicating no detectable damage of red blood cell membranes. The percentage of haemolysis (results not shown) was always less than 2.4%, data comparable to those of the blank. Moreover, no aggregation of erythrocytes was observed by a microscope after incubation with micelles (data not shown). Then, the absence of either lytic activity or aggregation of erythrocytes also supported a very good biocompatibility for either empty or BDP-loaded PHEA-PEG_{2000}-DSPE micelles.

Finally, to evaluate the capability of these systems to enhance the intracellular uptake of BDP, the effective amount of drug that penetrates into the 16HBE cells was determined. For this experiment, a maximum incubation time of 48 h and drug concentration of 7×10^{-5} M were chosen and, for comparison, the drug uptake from cells was also evaluated by using a drug suspension at the

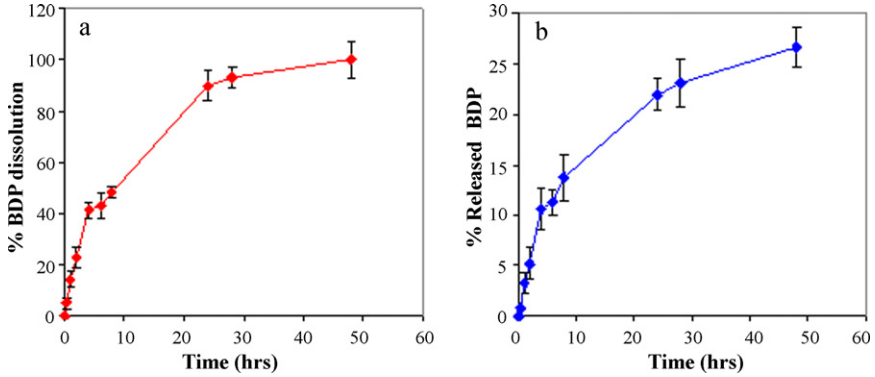


Fig. 7. (a) BDP diffusion profile across the dialysis membrane and (b) release profile of BDP from PHEA-PEG_{2000}-DSPE micelles in PBS (pH 7.4)/ethanol mixture. Data represent mean ± S.D. ($n = 3$).

same concentration; in effect, this kind of comparison was recently reported by other authors (Gaber et al., 2006).

Fig. 9 shows the internalized amount of BDP determined by HPLC in the 16HBE lysate after different incubation times (3, 6, 12, 24, 36 and 48 h) for cells treated with BDP, free or loaded into PHEA-PEG_{2000}-DSPE micelles.

As can be seen, a very high amount of drug (about 84 wt% of the initial amount incubated with cells) was detected into the cells after 48 h of incubation by using BDP-loaded PHEA-PEG_{2000}-DSPE micelles; on the contrary, a very small amount was detected by using drug suspension. However, it must be stressed that after 48 h incubation with the drug suspension, the cell viability resulted equal to 52%.

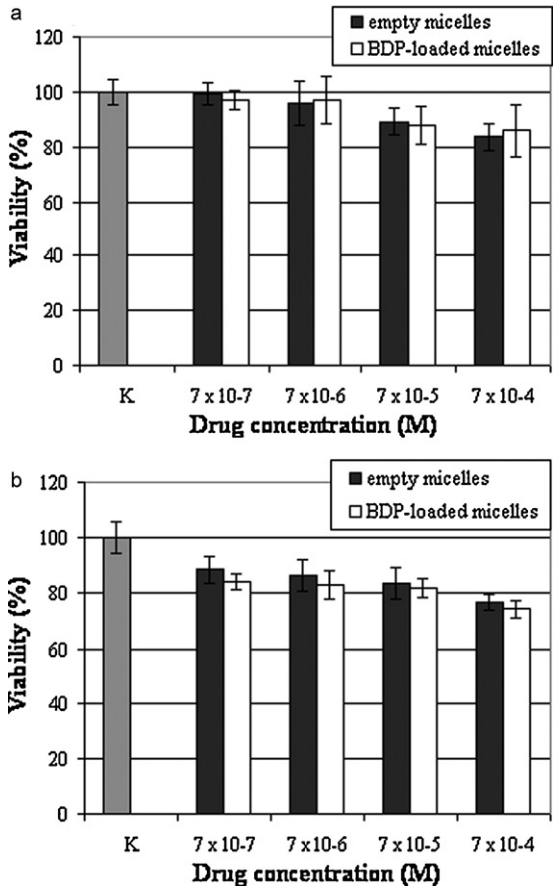


Fig. 8. 16 HBE cell viability after 24 (a) and 48 (b) h incubation with empty and BDP-loaded PHEA-PEG_{2000}-DSPE micelles (at different drug concentrations ranging between 7×10^{-7} and 7×10^{-4} M). The cell viability was determined by MTS colorimetric assay and the standard deviation values (±S.D.) were calculated on the basis of three experiments conducted in multiples of six.

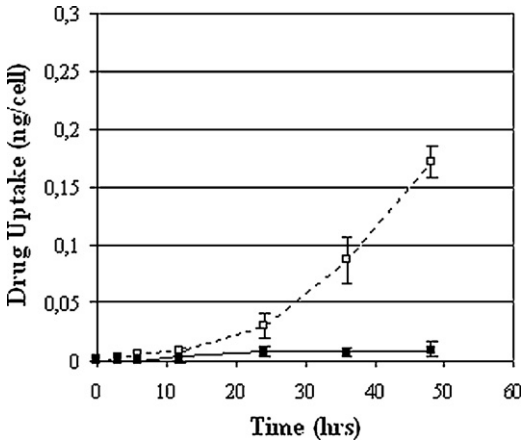


Fig. 9. Profile of BDP amount detected on lysate 16HBE cells as a function of incubation time by using BDP-loaded PHEA-PEG_{2000}-DSPE micelles (□) or BDP suspension (■).

These results suggest the hypothesis that a strong drug uptake could be promoted by the use of micelles. This enhanced drug uptake is probably due either to the endocytotic mechanism of micellar entry into cells or to the micelle action as cell penetrating enhancer on the released drug and present in the free form in the cell medium, this effect is expected to be beneficial for the treatment of tissue inflammation and asthma by maximizing therapeutic effect, and reducing drug dose and administrating frequency. Besides toxic effect due to systemic adsorption can be reduced. Moreover, relating to the peculiar chemical nature of our polymeric micelles, i.e. containing phospholipids, the presence of enhanced level of a mammalian secreted phospholipase A2 in an inflammatory site, that is able to degrade phosphatidyl ethanolamine residues, could increase the pulmonary drug release from the micelle core after cell internalization (Davidsen et al., 2001; Gaber et al., 2006; Vermehren et al., 2001).

4. Conclusions

In conclusion, a novel amphiphilic graft copolymer based on a pegylated phospholipid and a polyaspartamide was successfully synthesized by chemical grafting and by using disuccinimidyl carbonate. Obtained PHEA-PEG_{2000}-DSPE copolymer was properly characterized to confirm the amphiphilic structure, due to the hydrophilic shell based on both PHEA and PEG, and hydropho-

bic stearoyl tails belonging to phospholipids, and the absence of unreacted DSPE-PEG_{2000}-NH_2. The grafting of either hydrophilic or hydrophobic moieties such as PEG and stearoyl residues, respectively, on the PHEA backbone, performed in one single reaction, promotes, in aqueous phase, the copolymer arrangement into micelle-like structures at a CAC value of about 10^{-7} M.

PHEA-PEG_{2000}-DSPE micelles were capable to entrap hydrophobic drugs such as BDP and to increase its solubility of 240 folds. Both empty and drug-loaded micelles showed negative ζ potential values and a mean size in the nanometer scale, with differences related to the drug presence that increases the micelle core, but in any case mean size remained below 80 nm. The nanometer size of both empty and drug-loaded micelles was confirmed by FE-SEM analysis, which gives also morphology information about their spherical shape. Drug release studies showed a promising stability profile over 48 h, being micelles able to retain about 70% of the entrapped drug after 48 h. Excellent stability was also showed during storing in the dried form at 0 °C, being unchanged empty and drug-loaded micelles in terms of size, PDI and ζ potential. In vitro studies on human bronchial epithelium (16HBE) cells revealed the excellent biocompatibility of both empty and drug-loaded systems and the cellular internalization of BDP due to the incorporation into PHEA-PEG_{2000}-DSPE micelles.

Results encourage the use of these systems for the treatment of pulmonary diseases via topic administration as colloidal dispersion thanks to the capability of PHEA-PEG_{2000}-DSPE micelles to solubilize an adequate amount of BDP and to penetrate into epithelial cells of lung.

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References

Bai, S., Ahsan, F., 2009. Synthesis and evaluation of pegylated dendrimeric nanocarrier for pulmonary delivery of low molecular weight heparin. Pharm. Res. 26, 539–548.

Cavallaro, G., Licciardi, M., Giammona, G., Caliceti, P., Semenzato, A., Salmaso, S., 2003. Poly(hydroxyethylaspartamide) derivatives as colloidal drug carrier systems. J. Control. Release 89, 285–295.

Cavallaro, G., Maniscalco, L., Licciardi, M., Giammona, G., 2004. Tamoxifen-loaded polymeric micelles: preparation, physico-chemical characterization and in-vitro evaluation studies. Macromol. Biosci. 4, 1028–1038.

Courrier, H.M., Butz, N., Vandamme, T.F., 2002. Pulmonary drug delivery systems: recent developments and prospects. Crit. Rev. Ther. Drug Carrier Syst. 19, 425–498.

Craparo, E.F., Cavallaro, G., Bondì, M.L., Mandracchia, D., Giammona, G., 2006. Pegylated nanoparticles based on a polyaspartamide. preparation, physico-chemical characterization, and intracellular uptake. Biomacromolecules 7, 3083–3092.

Craparo, E.F., Ognibene, M.C., Casaletto, M.P., Pitarresi, G., Teresi, G., Giammona, G., 2008. Biocompatible polymeric micelles with polysorbate 80 for use in brain targeting. Nanotechnology 19, 485603 (12 pp.).

Craparo, E.F., Cavallaro, G., Ognibene, M.C., Teresi, G., Bondì, M.L., Giammona, G., 2009. Amphiphilic poly(hydroxyethylaspartamide) derivative-based micelles as drug delivery systems for ferulic acid. J. Drug Target. 17, 78–88.

Dabholkar, D.R., Sawant, R.M., Mongayt, D.A., Devarajan, P.V., Torchilin, V.P., 2006. Polyethylene glycol–phosphatidylethanolamine conjugate (PEG–PE)-based mixed micelles: some properties, loading with paclitaxel, and modulation of P-glycoprotein-mediated efflux. Int. J. Pharm. 315, 148–157.

Davidsen, J., Vermehren, C., Frokjaer, S., Mouritsen, O.G., Jørgensen, K., 2001. Drug delivery by phospholipase A(2) degradable liposomes. Int. J. Pharm. 214, 67–69.

Francis, M.F., Cristea, M., Winnik, F.M., 2004. Polymeric micelles for oral drug delivery: why and how. Pure Appl. Chem. 76, 1321–1335.

Gaber, N.N., Darwis, Y., Peh, K.K., Tan, Y.T.F., 2006. Characterization of polymeric micelles for pulmonary delivery of beclomethasone dipropionate. J. Nanosci. Nanotechnol. 6, 3095–3101.

Gao, J., Ming, J., He, B., Fan, Y., Gu, Z., Zhang, X., 2008. Preparation and characterization of novel polymeric micelles for 9-nitro-20(S)-camptothecin delivery. Eur. J. Pharm. Sci. 34, 85–93.

Giammona, G., Carlisi, B., Palazzo, S., 1987. Reaction of α,β-poly(N-2-hydroxyethyl)-DL-aspartamide with derivatives of carboxylic acids. J. Polym. Sci. Polym. Chem. Ed. 25, 2813–2818.

Goodwin, A.P., Tabakman, S.M., Welsher, K., Sherlock, S.P., Prencipe, G., Dai, H., 2009. Phospholipid–dextran with a single coupling point: a useful amphiphile for functionalization of nanomaterials. J. Am. Chem. Soc. 131, 289–296.

Helle, A., Hirsjärvi, S., Peltonen, L., Hirvonen, J., Wiedmer, S.K., Hyötyläinen, T., 2010. Novel, dynamic on-line analytical separation system for dissolution of drugs from poly(lactic acid) nanoparticles. J. Pharm. Biomed. Anal. 51, 125–130.

Klibanov, A.L., Maruyama, K., Torchilin, V.P., Huang, L., 1990. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett. 268, 235–237.

Ko, Y.T., Kale, A., Hartner, W.C., Papahadjopoulos-Sternberg, B., Torchilin, V.P., 2009. Self-assembling micelle-like nanoparticles based on phospholipid–polyethyleneimine conjugates for systemic gene delivery. J. Control. Release 133, 132–138.

Lasic, D.D., Woodle, M.C., Martin, F.J., Valentincic, T., 1991. Phase behavior of stealth-lipid lecithin mixtures. Period. Biol. 93, 287–290.

Lukyanov, A.N., Torchilin, V.P., 2004. Micelles from lipid derivatives of water-soluble polymers as drug delivery systems for poorly soluble drugs. Adv. Drug Deliv. Rev. 56, 1273–1289.

Marsh, D., Bartucci, R., Sportelli, L., 2003. Lipid membranes with grafted polymers: physicochemical aspects. Biochim. Biophys. Acta Biomemebr. 1615, 33–59.

Mendichi, R., Giammona, G., Cavallaro, G., Giacometti Schieroni, A., 2000. Molecular characterization of alpha,beta-poly(N-hydroxyethyl)-DL-aspartamide by light scattering and viscometry studies. Polymer 41, 649–8657.

Osada, K., Christie, R.J., Kataoka, K., 2009. Polymeric micelles from poly(ethylene glycol)-poly (amino acid) block copolymer for drug and gene delivery. J. R. Soc. Interface 6, S325–S339.

Pitarresi, G., Casadei, M.A., Mandracchia, D., Paolicelli, P., Palumbo, F.S., Giammona, G., 2007. Photocrosslinking of dextran and polyaspartamide derivatives: a combination suitable for colon-specific drug delivery. J. Control. Release 119, 328–338.

Rosen, M.J., 1989. Surfactants and Interfacial Phenomena. Wiley Interscience, New York.

Smola, M., Vandamme, T., Sokolowski, A., 2008. Nanocarriers as pulmonary drug delivery systems to treat and to diagnose respiratory and non respiratory diseases. Int. J. Nanomed. 3, 1–19.

Torchilin, V.P., 2007. Micellar nanocarriers: pharmaceutical perspectives. Pharm. Res. 24, 1–16.

Umland, S.P., Schleimer, R.P., Johnston, S.L., 2002. Review of the molecular and cellular mechanisms of action of glucocorticoids for use in asthma. Pulm. Pharmacol. Ther. 15, 35–50.

Vermehren, C., Jørgensen, K., Schiffelers, R., Frokjaer, S., 2001. Activity of mammalian secreted phospholipase A2 from inflammatory peritoneal fluid towards PEG–liposomes. Early indications. Int. J. Pharm. 214, 93–98.

Wang, Y., Wang, R., Lu, X., Lu, W., Zhang, C., Liang, W., 2010. Pegylated phospholipids-based self-assembly with water-soluble drugs. Pharm. Res. 27, 361–370.

Wilcox, J.B., Avery, G.S., 1973. Beclomethasone dipropionate corticosteroid inhaler: a preliminary report of its pharmacological properties and therapeutic efficacy in asthma. Drugs 6, 84–93.

Yang, Y.-T., Chen, C.-T., Yang, J.-C., Tsai, T., 2010. Spray-dried microparticles containing polymeric micelles encapsulating hematoporphyrin. AAPS J. 12, 138–146.