International Journal of Pharmaceutics 433 (2012) 16–24

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

PHEA-graft-polybutylmethacrylate copolymer microparticles for delivery of hydrophobic drugs

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ARTICLE INFO

Article history:
Received 27 January 2012
Received in revised form 20 April 2012
Accepted 21 April 2012
Available online 27 April 2012

Keywords:
Microparticles
PHEA-graft-copolymer
ATRP
Beclomethasone dipropionate
Flutamide

ABSTRACT

Polymeric microparticles encapsulating two model hydrophobic drugs, beclomethasone dipropionate (BDP) and flutamide (FLU) were prepared by using the high pressure homogenization-solvent evaporation method starting from a oil-in-water emulsion.

For the preparation of polymeric microparticles a α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) graft copolymer with comb like structure was properly synthesized via grafting from atom transfer radical polymerization (ATRP) technique, by using two subsequent synthetic steps. In the first step a polymeric multifunctional macroinitiator was obtained by the conjugation of a proper number of 2-bromoisobutyryl bromide (BIB) residues to the PHEA side chains, obtaining the PHEA-BIB copolymer. PHEA-BIB copolymer was then used as macroinitiator for the polymerization via ATRP of the hydrophobic monomer such as butyl methacrylate (BMA) to obtain the α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide-co-(N-2-ethylen-isobutyrate)-graft-poly(butyl methacrylate) copolymer (PHEA-IB-p(BMA)). Spherical microparticles with 1–3 microns diameter were prepared. Microparticles loaded with BDP or FLU were also prepared. In vitro mucoadhesion and enzymatic degradation studies evidenced bioadhesive properties and biodegradability of prepared microparticles, while release studies showed a different release profiles for the two loaded drugs: BDP was totally released from nanoparticles until 24 h in pulmonary mimicking conditions; differently a slower FLU release rate was observed in gastro-intestinal mimicking conditions. The in vitro cytotoxicity activity was assessed using 16HBE and Caco-2 cell lines. Results showed that exposure of both cell lines to BDP-loaded microparticles do not inhibited the cell growth; on the contrary FLU-loaded microparticles inhibited the cell growth, in particular of the Caco-2 cancer cell line, in a concentration- and time-dependent manner. Finally, uptake studies demonstrated that BDP-loaded microparticles and FLU-loaded microparticles effectively increased uptake of loaded drugs in a time-dependent manner, respectively on 16HBE and Caco-2 cell lines.

1. Introduction

Polymeric micro- and nanoparticles are being increasingly investigated for producing drug delivery systems able to give sustained drug release and targeting delivery. The advantages of using micro- or nanoparticulate drug delivery systems include: the possibility to maintain drug concentration in patient's blood and/or tissues at an active level for an extended time and a biodistribution and permeation through biological barriers and cellular uptake strictly dependent to the micron and submicron dimensions. These properties can be translated into an increased bioavailability of the encapsulated drug. In many cases, microencapsulation remains the most important formulation strategy for many bioactive substances, in particular for hydrophobic drugs (Anton et al., 2012; Wischke and Schwendeman, 2008; Purvis et al., 2006; Siepmann and Siepmann, 2006).

The principal requirement for fabricating controlled-release drug delivery systems is the availability of an appropriate material, which must be absolutely harmless to the organism and possess the necessary physical–chemical, mechanical and biomedical properties, including degradability in biological media; thus, the selection of the ideal polymer for microencapsulation is not immediate. While a wide variety of polymeric particulate carriers have been devised (Kumar, 2000) to protect active molecules from inactivation by the host and to control drug release in body fluids, a special attention should be paid to the biodegradability of polymers in order to prevent local or chronic toxicity that could be encountered after administration of non-biodegradable polymers

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http://dx.doi.org/10.1016/j.ijpharm.2012.04.052

(Wischke and Schwendeman, 2008). On another hand, the physico-chemical characteristics of the polymer used for microparticles production may also influence drug bioavailability. At this regard, for example, uptake of nanoparticles prepared from hydrophobic polymers seems to be higher than that obtained by particles with more hydrophilic surfaces, while more hydrophilic particles may be rapidly eliminated (Jung et al., 2000).

Microspheres and microparticles have been manufactured by various techniques, including solvent evaporation and phase separation (Wischke and Schwendeman, 2008), using non-solvent addition. One of the most simple and commonly employed method, is the high pressure homogenization-solvent evaporation method starting from a oil-in-water emulsion, that showed a good encapsulation rate of water insoluble compounds. For this reason, it was employed in the present study for producing polymeric microparticles encapsulating two model hydrophobic drugs, beclomethasone dipropionate and flutamide.

Beclomethasone dipropionate (BDP) is a synthetic chlorinated glucocorticoid diester, highly hydrophobic and thus, poorly soluble in water (49 μg/mL at 25 °C) (DrugBank, 2008). It is commonly used by inhalation in the treatment of asthma, and its therapeutic regimen in human generally recommends 3–4 doses of ≤200 μg daily, which indicates the relatively short local duration of action after administration.

Differently, flutamide (FLU) is a nonsteroidal antiandrogen. It exerts its antiandrogenic action by inhibiting androgen uptake and/or by inhibiting nuclear binding of androgen in target tissues or both. Being prostatic carcinoma an androgen-sensitive tumor, flutamide is primarily indicated in the treatment of this pathology. Like BPD, FLU is a poorly soluble in water drug (9.5 μg/mL at 25 °C) (DrugBank, 2008) and it has a serum half-life of about 6 h. Consequently the original dosing schedule for this medication is established at minimum 2 times daily (Murphy et al., 2004).

For the preparation of polymeric microparticles a α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) graft copolymer (PHEA-IB-p(BMA)) was properly synthesized via grafting from ATRP technique (Cavallaro et al., 2009), characterized and subsequently used to obtain microparticles by the high pressure homogenization-solvent evaporation technique.

The obtained microparticles have been evaluated in vitro in term of biodegradability, biocompatibility and mucoadhesivity properties in order to evaluate their use as drug delivery systems for pulmonary delivery of BDP and oral delivery of FLU.

2. Experimental

2.1. Materials and methods

α,β-Poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and purified according to the previously reported procedure (Giammona et al., 1987; Mendichi et al., 2000). Spectroscopic data (FT-IR and ^{1}H NMR) were in agreement with attributed structure (Giammona et al., 1987; Mendichi et al., 2000): ^{1}H NMR (300 MHz, D_2O, 25 °C, δ): 2.82 (m, 2H, –CH–**CH_2**–CO–NH–), 3.36 (t, 2H, –NH–**CH_2**–CH_2–OH), 3.66 (t, 2H, –CH_2–**CH_2**–OH), 4.72 (m, 1H, –NH–**CH**–CO–CH_2–). PHEA average molecular weight was 48.0 kDa (Mw/Mn = 1.66) based on PEO/PEG standards, measured by size exclusion chromatography (SEC). The SEC protocol involved using two Phenogel columns from Phenomenex (104R and 103R) connected to a Water 2410 refractive index detector and using a 0.01 M LiBr DMF solution as eluent with a flow of 0.8 mL/min. The column temperature was set at 50 °C. The ^{1}H NMR spectra were recorded in D_2O (Aldrich) using a Bruker Avance II 300 spectrometer operating at 300 MHz. Centrifugations were performed using a Centra MP4R IEC centrifuge. Sample centrifugations were performed at 4 °C and 8000 rpm for 10 min. Triethylamine (TEA), SEC polyethylene glycol standards, methanol (MeOH), polyvinyl pyrrolidone (PVP) 30 kDa were purchased from Fluka (Switzerland). 2-Bromoisobutyryl bromide (BIB), butyl methacrylate, 2,2′-bipyridine (bpy, 99%), copper(I) bromide (Cu^IBr 99.999%), dimethylacetamide (DMA), dimethylformamide (DMF), beclomethasone dipropionate (BDP) and flutamide (FLU) were purchased from Aldrich and were used as received. SpectraPor dialysis tubing was purchased from Spectrum Laboratories, Inc. (Italy).

2.2. Synthesis of α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide-co-(N-2-ethylen-isobutirrate)-graft-poly(butyl methacrylate) (PHEA-IB-p(BMA)) copolymer

Derivatization of PHEA with 2-bromoisobutyryl bromide (BIB) to obtain PHEA-BIB multifunctional macroinitiator was carried out using the protocol described previously (Cavallaro et al., 2009). The product was obtained with a yield of 95 wt.%, based on the starting PHEA. The degree of derivatization (DD), determined by ^{1}H NMR spectroscopy in D_2O and calculated according to the method reported elsewhere (Cavallaro et al., 2009), was 35 mol%.

The homopolymerization of butyl methacrylate, using PHEA-BIB as the macroinitiator, was carried out according to a previously reported procedure (Cavallaro et al., 2009), by modifying some reaction parameters. Briefly, the reaction of PHEA-BIB with butyl methacrylate (being molar ratio between butyl methacrylate and BIB residue equal to 10) was carried out in a previously degassed 1:1 DMF/water (v/v) solvent mixture at 50 °C for 20 h; Cu^IBr catalyst (25.5 mg, being the molar ratio between Cu^IBr and BIB linked group equal to 1) and bpy ligand (101 mg, being the molar ratio between bpy ligand and BIB linked group equal to 4) were then added to the flask under argon. Reaction was stopped by keeping reaction mixture in contact with air oxygen until the complete oxidation of copper. The reaction mixture was added drop-wise into double distilled water and the resulting solid residue was washed twice in a 1:1 H_2O/MeOH solvent mixture in order to eliminate the great part of unreacted butyl methacrylate and other reaction impurities. The white residue, obtained after centrifugation, was suspended in double distilled water and its purification was completed through exhaustive dialysis using a SpectraPor dialysis tubing with 12,000–14,000 molecular weight cutoff. After dialysis the suspension was freeze-dried from water. Obtained PHEA-IB-p(BMA) copolymer was characterized by ^{1}H NMR and spectroscopic data were in agreement with the previous results (Cavallaro et al., 2009).

2.3. Microparticle preparation from PHEA-IB-p(BMA)

Microparticles starting from PHEA-IB-p(BMA) graft copolymer were prepared by high pressure homogenization-solvent evaporation method. An organic phase was prepared by dispersing PHEA-IB-p(BMA) graft copolymer (typical concentration: 20 mg/mL) in chloroform. This organic solution was added to 20 mL of an aqueous phase, containing PVP 1.5% (w/v) and Pluronic F68 0.25% (w/v), and a primary o/w emulsion was obtained by using an Ultra-Turrax (T 25, Janke & Kunkel Ika-Labortechnik) for 20′ at 24,000 rpm. This emulsion was broken down into nano-droplets by applying external energy (500 bars, through a homogenizer) for six cycles. In particular, homogenization was performed using an EmulsiFlex^{TM}-C5 high pressure homogenizer (Avestin Inc., Canada), equipped with Totem CCS 338 (FIAC, Italy) air compressor. Then, the extraction of the solvent was achieved by evaporation under reduced pressure. As a consequence of this extraction, microparticle hardening occurred. Obtained microparticles were purified from PVP and Pluronic F68 by dialysis for 48 h. Finally,

microparticles were dried by using a Modulyo freeze-dryer (Labconco Corporation, Missouri, U.S.A.).

Analogous procedure was adopted for preparation of drug-encapsulated microparticles. In this case 180 mg of drug (BPN or FLU) were dissolved in chloroform before obtaining the primary emulsion.

2.4. Enzymatic degradation test of microparticles

Aliquots (10 mg) of PHEA-IB-p(BMA) based microparticles were incubated under continuous stirring (100 rpm) at 37.0 ± 0.1 °C with 2 mL of PBS pH = 7.4 containing esterase from porcine liver (final enzyme concentration ~280 U/mL) for 1 h, 8 h and 24 h. After each time samples of microparticles were purified by exhaustive dialysis, in order to eliminate low molecular weight polymer fractions released upon eventual microparticle degradation, and dispersions freeze-dried for further SEM analyses. A known amount of PHEA-IB-p(BMA) copolymer was also incubated with esterase under the same experimental conditions used for microparticles for 8 h; after this time dispersion was purified by dialysis, freeze-dried and the solid residue analyzed by ^{1}H NMR in order to evaluate the degradation effect on side chains of PHEA-IB-p(BMA) copolymer by esterase.

2.5. In vitro mucoadhesion test

Mucoadhesiveness of the microparticles was evaluated using the apparatus shown in Fig. 5 (Choy et al., 2008). A hydrophilic membrane (cellulose nitrate membrane, 0.45 μm pore size) was first soaked for 2 h in an aqueous mucin solution (0.1% mucin from porcine stomach, Type II). Then, 2 mL of a 10 mg/mL suspension of PHEA-IB-p(BMA) microparticles was applied dropwise at the center of the membrane. The membrane was then immediately washed with a continuous flow of saline phosphate buffer solution for 5 min at a rate of 10 mL/min and then dried under vacuum. The amount of adhered particles was determined by the weight difference between the mucin soaked membrane, previously dried and used as control, with the microparticles loaded membrane. Experiments were performed in triplicate. Adhered microparticles were also visualized by means of SEM analysis.

2.6. SEM analyses

For morphological studies, freeze-dried PHEA-IB-p(BMA) microparticle samples were observed by using an ESEM FEI Quanta 200F scanning electron microscope. Samples were dusted on a double-sided adhesive tape, previously applied on a stainless steel stub. Microparticles loaded on cellulose membrane used for mucoadhesion tests were analyzed as obtained after drying. All samples were sputter coated with gold prior to microscopy examination.

2.7. HPLC analyses

BDP and FLU were analyzed by HPLC using a Waters Breexe System Liquid Chromatograph equipped with a Waters 717 Plus Autosampler (40 μL injection volume), and a Shimadzu UV-VIS HPLC detector on line with a computerized workstation, monitored at 250 nm. Column: Reversed-phase Gemini C18 Phenomenex (5 μm, 4.6 × 250 mm column with a pre-column H5ODS-10CS). The used mobile phase was the same for both drugs: MeOH/PBS pH = 8.7 80:20, flow 1 mL/min.

2.8. Drug release from PHEA-IB-p(BMA) microparticles

Aliquots (5 mg) of BDP-loaded PHEA-IB-p(BMA) microparticles were dispersed in 0.5 mL of PBS at pH 7.4 and incubated at 37 ± 0.1 °C in the presence of carboxylesterase (Sigma, 485 U/mL) and lipase (Sigma, 650 μg/mL) under continuous stirring in a Benchtop 808C Incubator Orbital Shaker model 420. These conditions were adopted in order to mimic pulmonary mucosa environment.

Aliquots (10 mg) of FLU-loaded PHEA-IB-p(BMA) microparticles were dispersed in 2.5 mL of 0.1 N HCl solution and incubated for 2 h at 37 ± 0.1 °C in the presence of pepsin (Sigma, 2 mg/mL). Similarly, others FLU-loaded PHEA-IB-p(BMA) microparticles aliquots, dispersed in PBS at pH 6.8, were incubated at 37 ± 0.1 °C in the presence of pancreatin (from porcine liver, Sigma, 2 mg/mL) and bile salts (Sigma, 3 mM). These conditions were adopted in order to mimic gastro-intestinal environment.

At scheduled times, samples were filtered with cellulose acetate filters 0.2 μm and surnatant lyophilized. The residue obtained after freeze drying was dissolved in methanol (0.5 mL) and analyzed by HPLC in order to determine the released drug 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 microparticles. Data were correct taking in account the dilution procedure. Sink conditions were maintained during the experiments (drug concentration was always 10% below the maximum drug solubility). Each experiment was carried out in triplicate and the results were in agreement within ±5% standard error.

2.9. Cell viability assay on human epithelial (16HBE) and colon carcinona (Caco-2) cells

Cell viability was assessed by the MTS assay on 16HBE cell line (obtained as a generous gift from Istituto di Biomedicina e Immunologia Molecolare, Consiglio Nazionale delle Ricerche, Palermo, Italy) and on Caco-2 cell line (purchased from Sigma), at pH 7.4, using a commercially available kit (Cell Titer 96 Aqueous One Solution Cell Proliferation assay, Promega). Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10 vol% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL Amphotericin B (Sigma–Aldrich, Italy) under standardized conditions (95% relative humidity, 5% CO_2, 37 °C).

Cells were plated at a density of 1×10^5 cells mL^{-1} on a 96-well plate. Upon reaching confluence, cells were incubated with 10 μL per well of cell culture medium containing drug-loaded microparticles at a final drug concentration per well ranging between 7×10^{-4}–7×10^{-7} M. Moreover, cell viability was also carried out by incubating cells in the presence of empty microparticles, at concentrations equal to those used for drug loaded microparticle. After 4, 24 and 48 h of incubation, the growth medium was replaced with 100 μL of fresh MEM and 20 μL of a MTS/PMS solution were 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, as an average of 10 values. Cells incubated with the medium were used as control.

2.10. Cell drug uptake studies

For uptake studies, 16HBE cells were used for the evaluation of BDP-loaded microparticles uptake, and Caco-2 cells for the evaluation of FLU-loaded microparticles uptake.

In each experiment, 2×10^5 cells mL^{-1} were maintained in normal medium and 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, 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. After pre-incubation at 37 °C for 30 min, aliquots of drug-loaded microparticles or free drug suspensions were added to each well in order to obtain a final drug concentration equal to 7×10^{-5} M and incubated until 48 h. At different incubation periods (3, 6, 12, 24, 36 and 48 h), the medium was removed. The cell monolayer was washed twice with DPBS and the washings were combined with the incubation medium. 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 lyophilized. Drug uptake was quantified by analyzing the cell lysate and washing media by HPLC, after re-dispersion in methanol. It is noteworthy that the sum of internalized and not-internalized drug was always about 100% (data not reported). All experiment was carried out in triplicate for each incubation time.

2.11. Statistical analysis

Student's *t*-test was used to compare average values and determine statistically significant differences between the individual samples types. A one way analysis of variance (ANOVA) was used to evaluate group comparison. If the group by each time interaction was significantly different ($P < 0.05$), differences between groups were compared within an a posteriori Bonferroni *t*-test. All data were reported as mean values ± SD, unless otherwise stated.

3. Results and discussion

3.1. Synthesis and characterization of the hydrophobic PHEA-IB-p(BMA) copolymer

In this paper, polymeric microparticles potentially useful as delivery systems for hydrophobic drugs were prepared by using as starting copolymer PHEA-IB-p(BMA). The synthesis of this hydrophobic polyaspartamide-graft copolymer was based on the "grafting from" method, which consists in the in situ formation of side polymer chains from the polymer backbone (Börner et al., 2001).

The synthesis of α,β-poly(N-2-hydroxyethyl)-co-[N-2-ethylene(2-bromoisobutyrate)]-D,L-aspartamide (PHEA-BIB) used in this study have been performed as previously described in order to obtain the "multifunctional macroinitiator" for the subsequent grafting of butyl methacrylate in the side chains of PHEA (Cavallaro et al., 2009).

Experimental conditions adopted in this work allowed to obtain a PHEA-BIB copolymer with a side chain number equal to 30% mol/mol respect to PHEA repeating units. On BIB residues butyl methacrylate was polymerized by ATRP by using bpy/Cu complex as catalytic system, a monomer/BIB molar ratio of 10 and keeping the reaction mixture for 20 h at 50 °C.

In effect previously used reaction conditions, such as a lower reaction temperature (25 °C instead that 50 °C), even if for slightly longer time (24 h instead that 20 h), gave rise to shorter side polybutylmethacrylate chains, with a number of polymerization of about 2.8.

Reaction conditions and molecular parameters of synthesized PHEA-IB-p(BMA) copolymer are reported in Table 1.

The degree of derivatization (DD%) for the obtained PHEA-IB-p(BMA) copolymer, expressed as percentage of linked BMA moles for moles of repeating units of PHEA, was determined by ^{1}H NMR in DMSO-d_6, comparing the integral of the peak corresponding to protons at 0.90 ppm assigned to methyl groups belonging to linked BMA with the integral of the peak related to protons at 2.8 ppm assigned to CH_2, belonging to PHEA (Cavallaro et al., 2009). Moreover the mean number of repeating units of butyl methacrylate present in each side chain of PHEA-IB-p(BMA) copolymer was obtained by the ratio of DD% of linked butylmethacrylate residues (Table 1) and DD_{BIB}% of PHEA-BIB copolymer (DD_{BIB}% = 35 mol/mol).

As it can be seen according to data reported in Table 1, by increasing reaction temperature from 25 to 50 °C, the derivatization degree increases and, coherently, also the parameter *n*, i.e. the monomer mean number linked in the side chain (see Fig. 1 and Table 1), increases. Moreover, as the ^{1}H NMR spectrum of all synthesized copolymers does not show any peak assigned to protons belonging to unreacted BIB residues, we can conclude that in every obtained copolymers all BIB residues acted as polymerization propagation centers.

The chemical structure and schematic representation of PHEA-IB-p(BMA) are reported in Fig. 1.

The presence of hydrophobic chain, such as poly(BMA), on the PHEA backbone leads to a significant hydrophobicity increase of final PHEA-IB-p(BMA) graft copolymer and loss of water solubility. Due to its pronounced hydrophobicity PHEA-IB-p(BMA) resulted to be freely soluble in chlorinate solvents, such as $CHCl_3$, suggesting the possibility to arrange into solid aggregates in aqueous media. Hence, this copolymer was chosen as starting material for the preparation of polymeric microparticles by the high pressure homogenization-solvent evaporation method, a technique that permits to easily obtain monodisperse particles (Budhian et al. (2007).

3.2. Preparation and characterization of empty microparticles

Microparticle production process was performed by emulsifying a previously prepared organic phase, consisting of PHEA-IB-p(BMA) graft copolymer dissolved in chloroform, by vigorous stirring into an aqueous phase containing PVP and Pluronic F68. The obtained primary o/w emulsion was then broken down into micro-droplets by performing rapid passages under high pressure homogenization.

Then, the extraction of the solvent was achieved by evaporation under reduced pressure. As a consequence of this extraction, microparticle hardening occurred. Finally, obtained microparticles were purified by dialysis and freeze-dried. SEM images of freeze-dried microparticles are shown in Fig. 2.

As evidenced by SEM images, the obtained microparticles revealed an homogeneously spherical shape, a smooth surface and

Table 1
Reaction conditions and molecular parameters of PHEA-IB-p(ButMA) copolymers.

Reaction conditions	Reaction temperature (°C)	Reaction time (h)	DD (%)[b]	*n*[c]
Previous PHEA-IB-p(BMA)[a]	25	24	100	2.8
Current PHEA-IB-p(BMA)	50	20	230	6.6

[a] As used in Cavallaro et al. (2009).
[b] DD% = (linked ButMA residues/PHEA repeating units) × 100 (mol/mol).
[c] *n*, mean monomer residue for each chain.

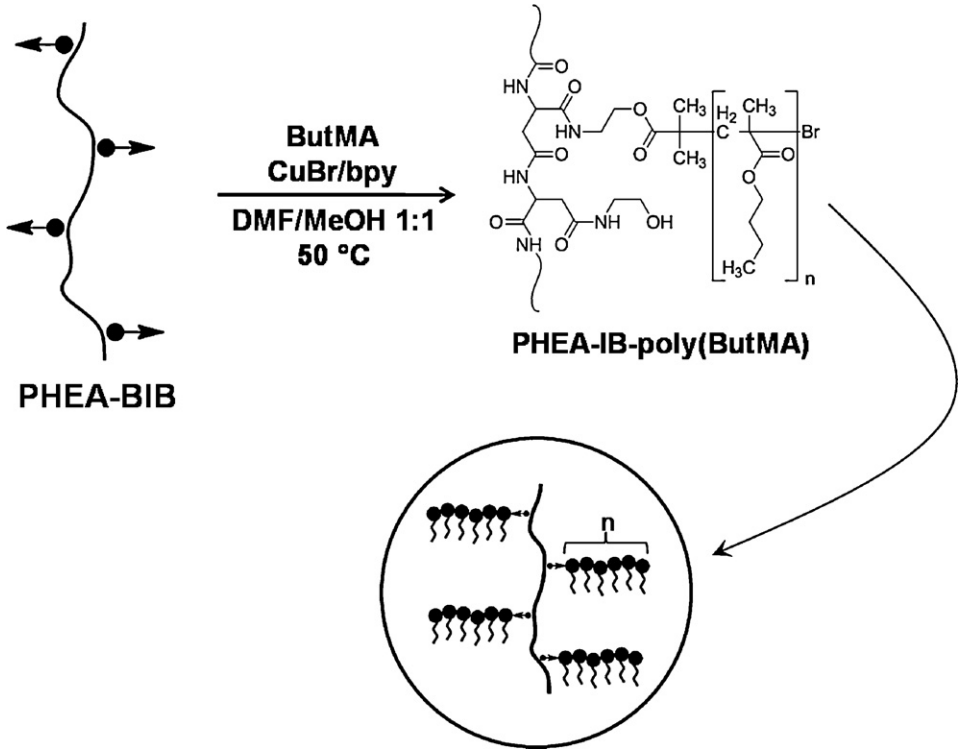

Fig. 1. Structure and schematic representation of PHEA-IB-p(BMA).

mean diameter values ranging from 1 to 3 μm (average particles size 1.5 μm).

Because of the presence of hydrolizable bonds in the structure of PHEA-IB-p(BMA) graft copolymer (i.e., ester groups), their stability after enzymatic treatments was investigated. In particular, the enzymatic stability of the samples was evaluated by incubating microparticles in PBS pH = 7.4 containing esterase at 37 °C for 24 h. At scheduled times (1 h, 8 h and 24 h) samples were properly treated (see experimental part) and analyzed by SEM, in order to highlight eventual morphological and dimensional alterations. Actually, enzyme can act on two site of action into copolymer structure: (a) on ester bonds between polybutylmethacrylic chains and PHEA polymer backbone and (b) on ester bonds of butyl residues inside polybutylmethacrylic chains. As evidenced by SEM reported in Fig. 3, esterase exerts, at short time (1 h and 8 h), only a modest effect on microparticle surface. On the contrary, after 24 h incubation time it is possible to observe microparticle deformation with fusion among them. However enzymatic degradation observed for all samples seems due to enzyme action on ester bonds of microparticle surface. On the other hands the hydrolytic effect of esterase on the side chains of PHEA-IB-pBMA copolymer was confirmed by ^{1}H NMR analyses of this copolymer after incubation for 8 h with esterase (data not shown).

The evaluation of mucoadhesive properties of polymeric carriers is another important characterization parameter, strictly related to adsorption and bioavailability of encapsulated drug. In fact, microparticles able to remain adherent to the mucosa surface for an extended time should exhibit sustained release properties as a consequence of the reduced loss caused by mucosal drainage, hence increasing drug bioavailability. At this purpose PHEA-IB-p(BMA) microparticle mucoadhesiveness was evaluated by using a procedure that consisted into the examination of microparticle adhesion on a cellulose membrane soaked with mucine, to simulate the pulmonary epithelia surface, after washing

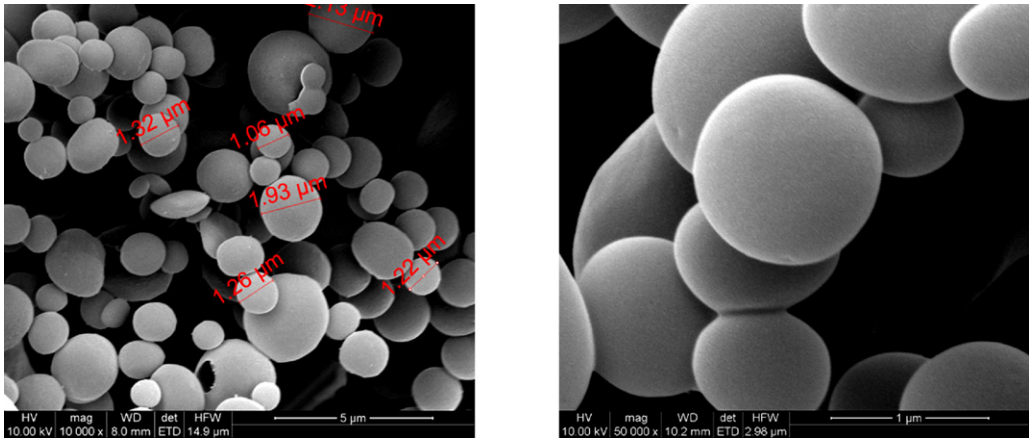

Fig. 2. SEM images of empty PHEA-IB-p(BMA) microparticles.

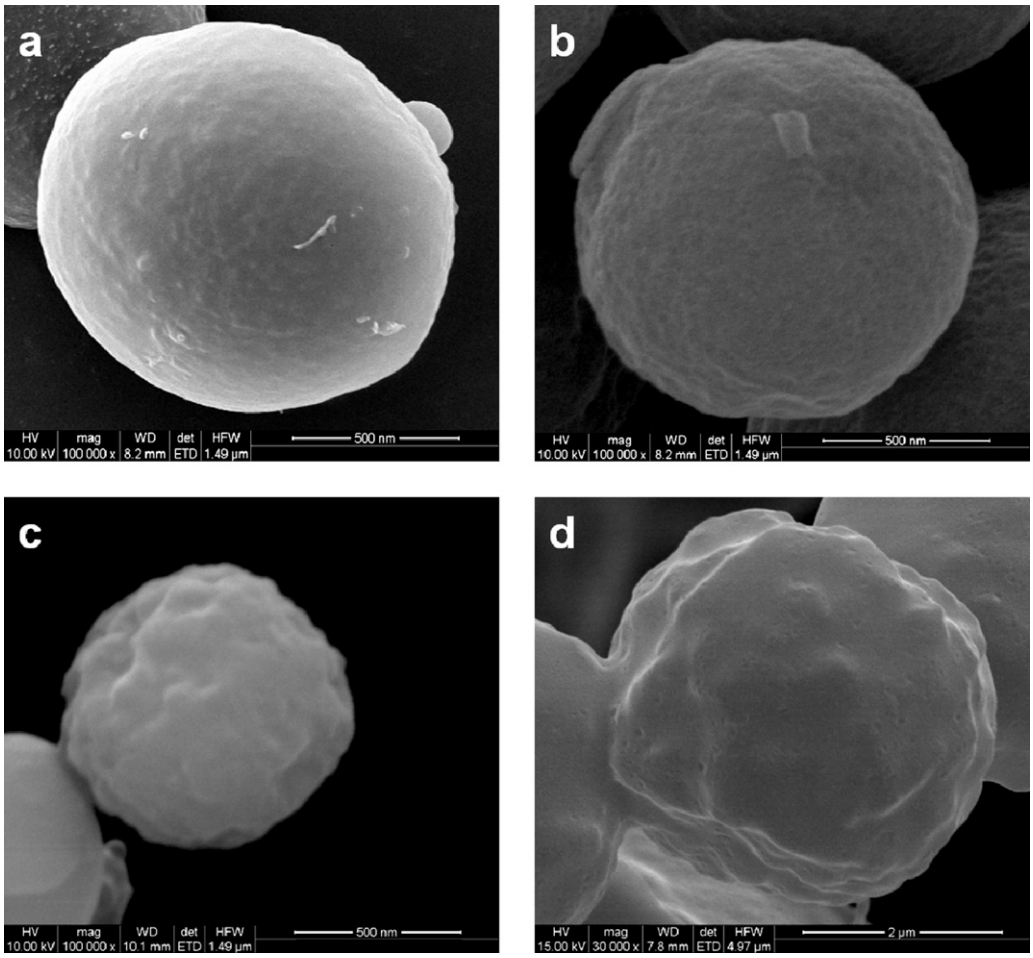

Fig. 3. SEM images of untreated PHEA-IB-p(BMA) microparticles (a) and treated PHEA-IB-p(BMA) microparticles after 1 h (b), 8 h (c) and 24 h (d) incubation time in the presence of esterase.

particles applied on the mucous membrane with saline solution, as shown in Fig. 4. Under these conditions, about 80 wt.% of applied PHEA-IB-p(BMA) microparticles remained adherent to the mucous membrane.

SEM analysis of the these membranes loaded with PHEA-IB-p(BMA) microparticles confirmed that a high amount of microparticles remained adherent to the membrane (Fig. 5), demonstrating the good ability of these microparticles to potentially adhere to the pulmonary epithelia surface.

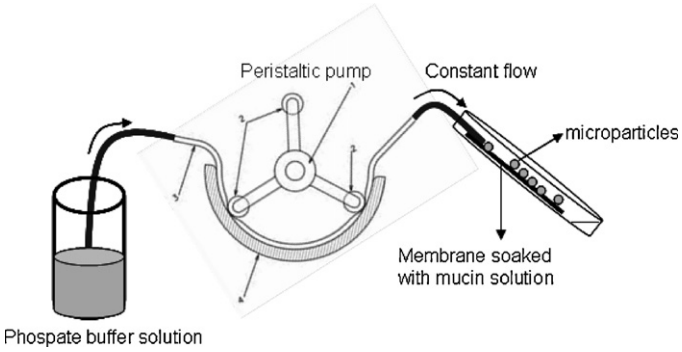

Fig. 4. Schematic drawing of the apparatus used for the mucoadhesiveness evaluation.

3.3. Preparation and characterization of drug loaded microparticles

PHEA-IB-p(BMA) microparticles containing BDP and FLU were prepared in order to investigate their potential use as delivery systems for hydrophobic drugs. At this purpose, drug molecules with different structure were used to understand if different nature and physico-chemical properties of the drug may influence loading efficiency and microparticle applications. The obtained drug-loaded microparticles were characterized in terms of drug loading (DL%), morphology and drug release rate.

Drug loading procedure was carried out during the microparticles formation process, by dissolving proper amount of drug in the organic phase, in which it resulted to be freely soluble. To quantify the amount of BDP or FLU loaded in PHEA-IB-p(BMA) microparticles, an HPLC analysis was carried out, as reported in the experimental part. The DL%, expressed as weight percentage ratio between the loaded drug and the dried system (microparticles + drug), resulted to be 8% and 4% (w/w) for BDP and FLU, respectively; in effect considering different molecular weight of two drugs (521.04 for BDP versus 276.22 for FLU) the loading capacity of PHEA-IB-p(BMA) microparticles of two drugs is quite similar. This result suggests that, despite different structure of two molecules no difference in loading capacity of microparticles was observed under the used experimental conditions.

Successively, drug-loaded microparticles were characterized in terms of morphology in order to evaluate eventual effect of the drug presence on the microparticle formation process. Thus, obtained

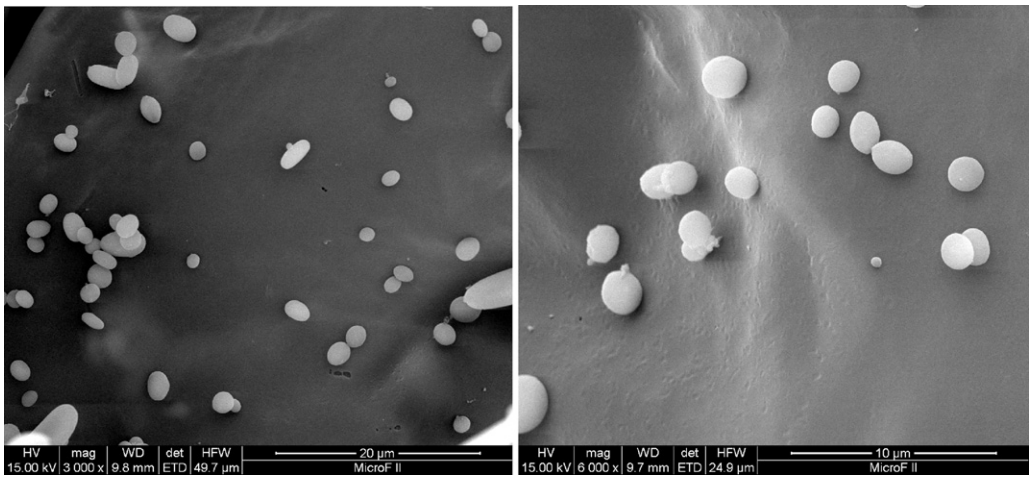

Fig. 5. SEM images of the mucous membranes loaded with PHEA-IB-p(BMA) microparticles.

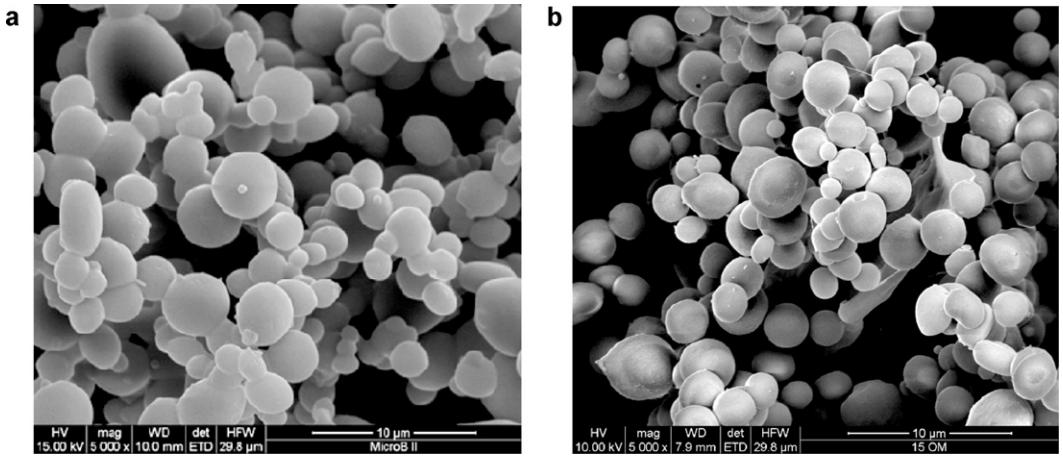

Fig. 6. (a) SEM image of BDP-loaded PHEA-IB-p(BMA) microparticles. (b) SEM images of FLU-loaded PHEA-IB-p(BMA) microparticles.

freeze-dried microparticles were characterized by SEM (Fig. 6a and b). No significant increase in mean diameter of drug-loaded microparticles (average particles size 1.61 μm) was detected in comparison with empty microparticles.

To evaluate the ability of PHEA-IB-p(BMA) microparticles to release loaded drug, release studies were carried out by using different release conditions related to the encapsulated drug and the administration route hypotized for two different systems. In particular, a medium containing carboxylesterase and lipase in PBS at pH 7.4 was adopted in order to mimic pulmonary mucosa environment for BDP release studies; differently, 0.1 N HCl solution in the presence of pepsin (for the first 2 h) and PBS at pH 6.8 in the presence of pancreatin and bile salts (for the successive 22 h) were adopted in order to mimic gastro-intestinal environment for FLU release studies. In Figs. 7 and 8 are reported the release profile of these two drugs into the proper experimental conditions.

As can be seen, a significant difference in the release profile of BDP and FLU was observed for two systems [BDP loaded-PHEA-IB-p(BMA) microparticles and FLU loaded-PHEA-IB-p(BMA) microparticles] under two different experimental conditions. Actually, for PHEA-IB-p(BMA) microparticles containing BDP, designed for pulmonary delivery, release rate was quite high and release was completed within 24 h, with about 80% of loaded BDP released within first 8 h. On the contrary, from FLU loaded-PHEA-IB-p(BMA) microparticles, drug release rate was slower with only about 30% of loaded FLU released from microparticles after 24 h of incubation. Therefore taking into account that in the presence of esterase studied microparticulate systems undergo degradation (see previously showed data), drug release from these microparticles can be explained evoking the occurring of both mechanism of diffusion of the drug through the polymer matrix and matrix degradation.

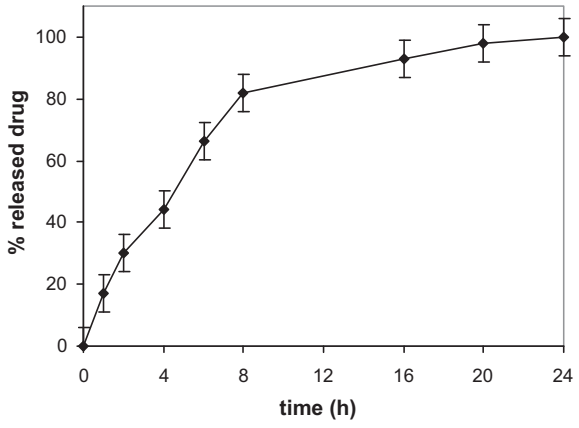

Fig. 7. BDP release from PHEA-IB-p(ButMA) from 0 to 24 h in pulmonary mimicking conditions.

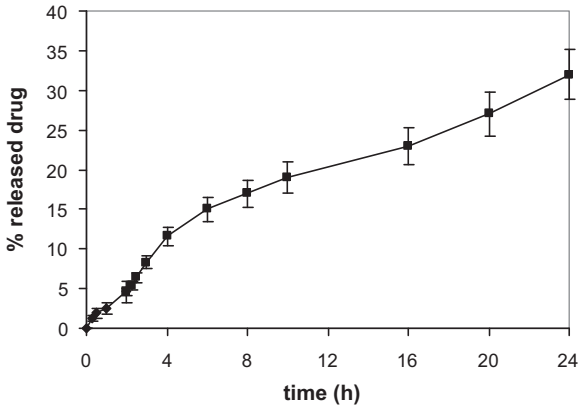

Fig. 8. FLU release from PHEA-IB-p(ButMA) from 0 to 10 h in gastro-intestinal mimicking conditions.

3.4. In vitro biological evaluation of PHEA-IB-p(BMA) microparticles

Cytotoxicity of BDP-loaded and FLU-loaded microparticles on 16 HBE and Caco-2 cell lines was evaluated by the MTS assay at different drug concentrations (7×10^{-4}–7×10^{-7} M), after 4, 24 and 48 h incubation times. Moreover, cell viability was also evaluated

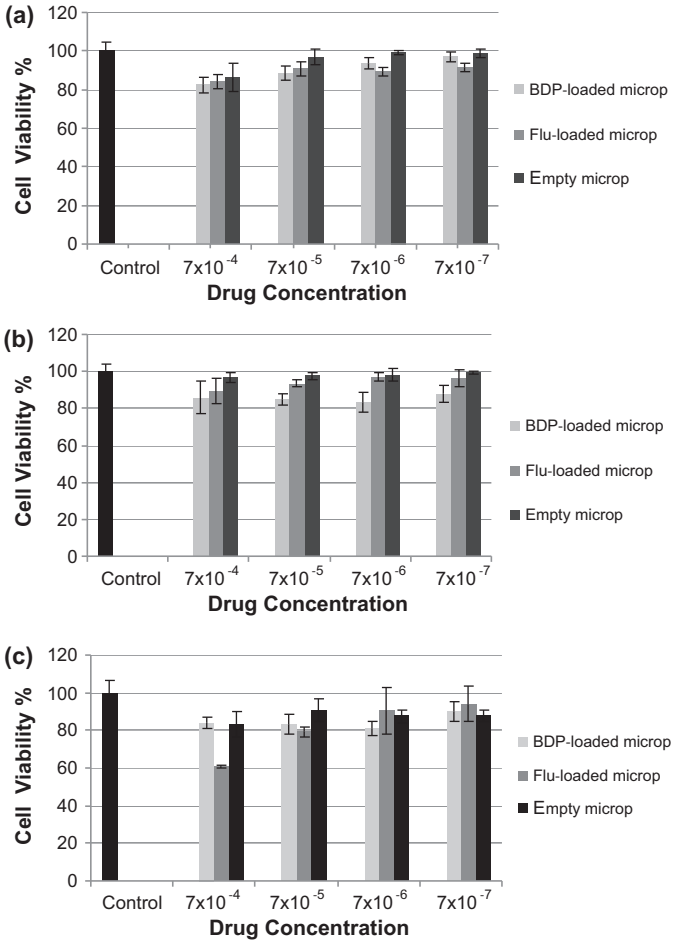

Fig. 9. 16 HBE cell viability after 4 (a), 24 (b) and 48 h (c) incubation with empty, BDP-loaded and FLU-loaded microparticles (at different drug concentrations ranging between 7×10^{-4} and 7×10^{-7} M). The standard deviation values (±SD) were calculated on the basis of three experiments conducted in multiples of six. Cells incubated with the medium were used as control.

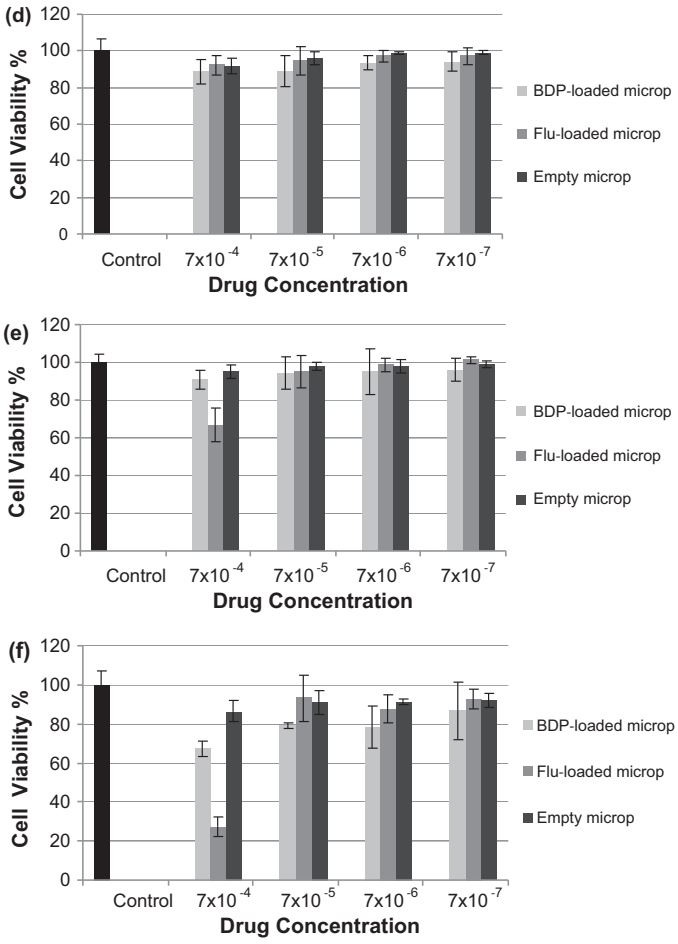

Fig. 10. Caco-2 cell viability after 4 (d), 24 (e) and 48 (f) h incubation with empty, BDP-loaded and fluta-loaded microparticles (at different drug concentrations ranging between 7×10^{-4} and 7×10^{-7} M). The standard deviation values (±SD) were calculated on the basis of three experiments conducted in multiples of six. Cells incubated with the medium were used as control.

in the presence of empty microparticles, at concentrations equal to those used for drug-loaded microparticles. Results are reported in Figs. 9 and 10.

Results evidenced that both empty and FLU or BDP-loaded microparticles showed lack of cytotoxicity at concentrations ranging between 7×10^{-4} and 7×10^{-7} M, after 4, 24 and 48 h incubation on the normal epithelial cell line (16HBE). At the highest tested drug concentration (7×10^{-4} M), only FLU-loaded microparticles resulted slightly toxic, being viability values around 60%. This result can be explained considering the high used drug concentration.

Differently on the cancer cell line (Caco-2) cell viability resulted drastically reduced by FLU-loaded microparticles as a function of time, being around 30% after 48 h of incubation. In this last case, result is consistent with apoptotic effect induced by FLU on cancer cells (Zhang et al., 2005).

Finally, in order to evaluate the effective amount of drug that penetrates into the cells thanks to the incorporation into these microparticles, cell uptake experiments were conducted by using 16HBE cells for BDP-loaded microparticles and Caco-2 cells for FLU-loaded microparticles. For this experiments, a maximum incubation time of 48 h and drug concentration of 7×10^{-5} M were chosen. For comparison, the drug uptake from cells was also evaluated by using a drug suspension at the same concentration (Craparo et al., 2011); however after 48 h incubation with the drug suspensions at the same

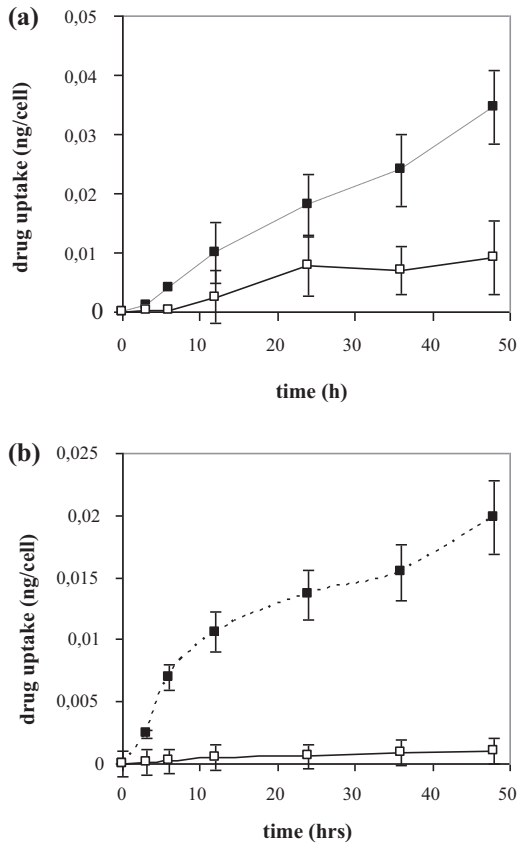

Fig. 11. Internalized amount (ng/cell) of BDP into 16HBE (a) and FLU into Caco-2 (b) cells, respectively, determined by HPLC at different incubation times (3, 6, 12, 24, 36 and 48 h) for cells treated with drug suspension (□) or drug-loaded microparticles (■).

concentration (7×10^{-5} M), the cell viability resulted above 80% (data not reported).

Fig. 11a and b showed the internalized amount of BDP into 16HBE and of FLU into Caco-2 cells, respectively, determined by HPLC in the cellular lysate after different incubation times (3, 6, 12, 24, 36 and 48 h) for cells treated with drug suspension or drug-loaded into microparticles.

In both examined cases, cell drug uptake was increased when drug was loaded into PHEA-IB-p(BMA) microparticles, respect to free drugs. The internalized drug amount can be quantified in about 80 wt.% of the starting BDP amount incubated with 16HBE cells and about 60 wt.% of the starting FLU amount incubated with Caco-2 cells, respectively; on the contrary, a very small amount of internalized drug was detected when drug suspension was used.

These results suggest the hypothesis that PHEA-IB-p(BMA) microparticles can be able to interact with cell membrane and promote the uptake of encapsulated drug, upon microparticles endocytosis and drug diffusion. The enhanced drug uptake could be related also to microparticle adhesion on cell membranes attributable also to the hydrophobic structure of PHEA-IB-p(BMA) copolymer constituting microparticles.

4. Conclusions

Alpha,beta-poly(N-2-hydroxyethyl)-D,L-aspartamide-co-(N-2-ethylen-isobutirrate)-graft-poly(butyl methacrylate) (PHEA-IB-p(BMA)) polymeric microparticles, encapsulating two model hydrophobic drugs, beclomethasone dipropionate (BDP) and flutamide (FLU), were produced employing solvent-evaporation microencapsulation method. For this purpose PHEA-IB-p(BMA) hydrophobic copolymer was synthesized via grafting from ATRP technique, by using PHEA-BIB as polymeric multifunctional macroinitiator for the polymerization via ATRP of butyl methacrylate (BMA) monomer. Spherical microparticles with 1–3 microns diameter were obtained, having mucoadhesive properties and biodegradability as tested by in vitro experiments. Release studies showed a different release profiles for the two encapsulated drugs: BDP was totally released from nanoparticles until 24 h in pulmonary mimicking conditions; differently a slower FLU release rate was observed in gastro-intestinal mimicking conditions, being release about 30% of encapsulated flutamide after 24 h.

Both empty and FLU or BDP-loaded microparticles showed high biocompatibility on normal 16HBE epithelial cell line. At the highest tested drug concentration (7×10^{-4} M), only FLU-loaded microparticles showed a significant cytotoxic activity against Caco-2 cancer cell line, in measure higher than free flutamide; therefore this result is consistent with the ability of PHEA-IB-p(BMA) microparticles to increase uptake of the loaded drugs into Caco-2 (for FLU) cell lines. The ability of PHEA-IB-p(BMA) microparticles to increase cell drug uptake was detected also for BDP on HBE cells.

These results are consistent with the hypothesis to propose these microparticles as drug delivery systems able to maximizing cell drug entry and therefore therapeutic effect and reduce drug dose and administration frequency either for the pulmonary administration of BDP or for oral administration of FLU.

Acknowledgment

Authors thank MIUR for funding. Authors thank Dr. Paolo Guerra, from the Department of Ingegneria Chimica, Gestionale, Informatica, Meccanica, University of Palermo, for technical support during SEM analysis.

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