RESEARCH ARTICLE

Synthesis, Characterization and *in vitro* Anti-Tumoral Evaluation of Erlotinib-PCEC Nanoparticles

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Abstract

Background: Development of a nanosized polymeric delivery system for erlotinib was the main objective of this research. Materials and Methods: Poly caprolactone-polyethylene glycol-polycaprolactone (PCEC) copolymers with different compositions were synthesized via ring opening polymerization. Formation of triblock copolymers was confirmed by HNMR as well as FT-IR. Erlotinib loaded nanoparticles were prepared by means of synthesized copolymers with solvent displacement method. Results: Physicochemical properties of obtained polymeric nanoparticles were dependent on composition of used copolymers. Size of particles was decreased with decreasing the PCL/PEG molar ratio in used copolymers. Encapsulation efficiency of prepared formulations was declined by decreasing their particle size. Drug release behavior from the prepared nanoparticles exhibited a sustained pattern without a burst release. From the release profiles, it can be found that erlotinib release rate from polymeric nanoparticles is decreased by increase of CL/PEG molar ratio of prepared block copolymers. Based on MTT assay results, cell growth inhibition of erlotinib has a dose and time dependent pattern. After 72 hours of exposure, the 50% inhibitory concentration (IC50) of erlotinib hydrochloride was appeared to be 14.8 µM. Conclusions: From the obtained results, it can be concluded that the prepared PCEC nanoparticles in this study might have the potential to be considered as delivery system for erlotinib.

Keywords: Erlotinib - PCEC - solvent displacement method - nanoparticles

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Introduction

Erlotinib is a potent and selective tyrosine kinase inhibitor. It reversibly binds to the binding site of adenosine triphosphate (ATP) in tyrosine kinase domain of epidermal growth factor receptors and inhibits auto phosphorylation of tyrosine kinase (Gale, 2003; Aydiner et al., 2013). Inhibition of tyrosine kinase leads to apoptosis promotion, inhibition of angiogenesis and finally preventing excessive cell proliferation (Clay et al., 2005; Smith, 2005; Makrilia et al., 2009). Erlotinib which is used in treatment of various solid tumors such as non-small cell lung cancer is available in oral form (Clay et al., 2005; Smith, 2005; Qi et al., 2012). Oral bioavailability of erlotinib was obtained 59% and 76% respectively in healthy volunteers and cancer patients (Ranson et al., 2010). No data are available regarding the marketing of other formulation type of this drug. Development of new delivery systems for erlotinib is limited by its poor solubility. Nevertheless reverse micelle-loaded lipid nanocarriers containing erlotinib hydrochloride were produced by Vrignaud et al. recently (Vrignaud et al., 2012). Moreover Marslin et al. found that PLGA nanoparticles containing erlotinib hydrochloride demonstrated less sub-acute toxicity than free drug in rats (Marslin et al., 2009). In another study conducted by Xu et al. erlotinib loaded multifunctional magnetic nanoparticles was prepared (Xu et al., 2012).

Pathophysiological characteristics of solid tumors including extensive angiogenesis, defective vascular architecture, impaired lymphatic drainage and greatly increased production of a number of permeability mediators, result in the enhanced permeability and retention (EPR) effect. Thus this effect makes drug loaded nano-carriers to concentrate in tumor sites (Maeda et al., 2000; Kingsley et al., 2006; Vicent and Duncan, 2006; Yadav et al., 2014). Among particulate drug carriers, polymeric nanoparticles exhibit suitable characteristics for encapsulation of many drugs (Torchilin, 2007). Nevertheless some of the essential properties of polymers for biomedical application such as drug delivery systems are biodegradability, biocompatibility, suitable solubility and appropriate mechanical properties. Polyesters belong to the hydrolytically degradable polymers which are prepared by ring opening polymerization. Poly glycolic acid, poly lactic acid and poly caprolactone (PCL) are the most extensively investigated polyesters (Okada, 2002).

Poly caprolactone (PCL) is a semi-crystalline, hydrophobic polymer with a glass transition temperature

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(Tg) of −60°C and melting point ranging from 59 to 64°C (Nair and Laurencin, 2007). PCL is synthesized by the ring-opening polymerization method with using ε-caprolactone monomer and a variety of anionic, cationic and co-ordination catalysts (Okada, 2002). Because of excellent biocompatibility, good solubility and low melting point, PCL is suitable for controlled drug delivery. But biodegradation of PCL is slow which restricts its clinical application. In order to overcome this issue, preparation of PCL copolymers is proposed (Merkli et al., 1998; Freiberg and Zhu, 2004; Sinha et al., 2004). Block and random copolymers of PCL can be synthesized by using monomers such as ethyleneoxide, polyvinylchloride, chloroprene, polyethylene glycol(PEG), polystyrene, diisocyanates (urethanes), tetrahydrofuran (THF), diglycolide, dilactide, δ-valerlactone, substituted caprolactones, 4-vinyl anisole, styrene, methyl methacrylate and vinyl acetate (Okada, 2002; Woodruff and Hutmacher, 2010). Among these monomers, PEG is suitable to form caprolactone block copolymers because of its hydrophilicity, nontoxicity and absence of antigenicity and immunogenicity (Wei et al., 2009). PCL and its copolymers were utilized to develop nanoparticles containing various drugs (Sinha et al., 2004; Dubey et al., 2012; Pereira Ade et al., 2013; Yin et al., 2013). For instance tamoxifen loaded Poly ethylene oxide-modified poly caprolactone nanoparticles were prepared by Shenoy et.al which demonstrated tumor-selective biodistribution (Shenoy and Amiji, 2005). Poly(caprolactone)-poly(ethyleneoxide) (PCE), methoxy polyethylene glycol polycaprolactone (MePEG/PCL), polycaprolactone- polyethylene glycol- poly caprolactone (PCEC) block copolymers are different types of CL block copolymers which used in encapsulation of bovine serum albumin(BSA), taxol and clonazepam respectively (Lu et al., 1999; Ryu et al., 2000; Kim and Lee, 2001). The purpose of present study was preparation and physicochemical characterization of erlotinib nanoparticles by using synthesized three block PCEC copolymers. We prepared erlotinib loaded nanoparticles by means of solvent displacement method. In this method solution of a polymer in a water-miscible solvent is introduced to the aqueous medium in the presence or absence of a surfactant. Fast diffusion of organic solvent leads to precipitation of polymer and nanospheres formation (Quintanar-Guerrero et al., 1998; Pinto Reis et al., 2006). Also we investigated cytotoxicity of free drug and drug loaded nanoparticles by MTT assay.

Materials and Methods

Materials

Erlotinib hydrochloride was synthesized as previously described (Barghi et al., 2012). PEG with average molecular weight of 4KDa and caprolactone monomer was purchased from Merck chemical company (Germany). Stannous octoate was obtained from Alfa Aesar, A Johnson Matthey Company (Germany). Poloxamer 188 was purchased from BASF (Germany). Fetal bovine serum (FBS) and RPMI were obtained from Gibco (Germany). MTT (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide) was purchased from Sigma Aldrich (USA). All used solvents were HPLC grade and purchased from Merck chemical company (Germany).

Synthesis and characterization of PCEC

PCEC copolymers with various molar CL/PEG ratios (70, 280, 560 and 840) were synthesized by ring opening polymerization of epsilon caprolactone and PEG in presence of stannous octoate as catalyst. Polymerization of PCEC was carried out in a two necked vessel equipped with a stirrer, a thermometer and a gas inlet tube. Calculated amount of epsilon-caprolactone, PEG with average molecular weight of 4 KDa and stannous octoate were introduced into a two necked vessel. The reaction mixture was stirred under dry nitrogen for 6 hours at 130°C. After completion of polymerization; the vessel was connected to a vacuum system for 30 minutes at 180°C (Liu et al., 2008). After cooling of reaction mixture to the room temperature, the synthesized copolymer was dissolved in dichloromethane and then isolated by precipitation with n-hexane. The obtained solid material was filtered and the residual solvent was removed under reduce pressure. Fourier-transform infrared spectroscopy of PEG and synthesized copolymers were obtained on a Bomem 2000 FT-IR system (Bomem, Quebec, Canada). Copolymers were dissolved in dichloromethane and thin film of this solution was casted on NaCl plate. 1HNMR spectra of copolymers in CDCl3 were obtained with a Bruken-Spectrospin 400 MHz spectrometer (Varian, Switzerland). Thermal behavior of copolymers was recorded on a DSC-60 (Shimadzu, Kyoto, Japan). Thermogram of the samples was obtained at a scanning rate of 10°C/min covering temperature range of 25–200°C.

Preparation of nanoparticles

Erlotinib nanoparticles were prepared via solvent displacement method. Drug and polymer (drug/polymer ratio: 1/10) were dissolved in a mixture of acetone and methanol (methanol/acetone ratio: 1/6) and then this organic solution was added dropwise to 50 ml of aqueous phase containing poloxamer 188 (1.5-3%) by using homogenizer at 20000 rpm. Finally solvents were evaporated under vacuum homogenization. Resulting nanoparticles were collected by ultrafiltration device (Amicon Ultra-15, 100KD) and then lyophilized.

Particle size and morphology evaluation

Particle size and size distribution were determined by means of laser diffraction particle size analyzer (Sald 2101, Shimadzu, Japan). The morphology of nanoparticles was observed by Transmission Electronic Microscopy (TEM) (LEO 906, Germany). A drop of nano-suspension was placed on copper grid and dried overnight before observation.

Surface charge determination

Zeta potential of nanoparticles was measured by Malvern zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). The lyophilized nanoparticles were dispersed in distilled water in order to determine their surface charge.

Encapsulation efficiency

After preparation of erlotinib nanosuspension, nanoparticles were separated from the aqueous medium by ultrafiltration with using Amicon ultra centrifugal filters (100 KDa molecular weight cut-offs). Aqueous medium was diluted with methanol (50:50) and concentration of erlotinib in this solution was determined spectrophotometrically at 339.8 nm using the UV-visible spectrophotometer (Shimadzu, Japan). Amounts of entrapped drug in nanoparticles were defined as a difference between total amount of used erlotinib in nanoparticles preparation process and the amount of erlotinib exist in aqueous medium.

In vitro drug release

The lyophilized erlotinib loaded nanoparticles were dispersed in 5 ml of dissolution medium (phosphate buffered saline (PBS) at pH 7.4, containing 0.02% soy lecithin) and put into a dialyzer with polycarbonate membrane (pore size: 50 nm).The dialyzer was immersed in100 ml of dissolution medium which stirred at 100 rpm, 37°C. At specific time intervals, 1 ml samples were taken out for analysis and replaced with same volume of fresh medium.

Analyzing of samples was carried out by using HPLC (Knauer, Germany). A reversed-phase Symmetry C18 column, (Shim-pack VP ODS, 250 mm × 4.6 mm, 5 μm) was used for analysis of erlotinib. The flow rate of mobile phase (45% (v/v) acetonitrile, 40% (v/v) potassium dihydrogen phosphate buffer (pH=4.5) and 15% (v/v) methanol) was 1.3 ml/min. Erlotinib was detected at 332 nm with an ultraviolet detector (Bolandnazar et al., 2013).

Cell viability assay

Human non–small-cell lung cancer cell line (A549, Pasteur Institute, Iran) were grown in RPMI 1640 containing 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C. An MTT colorimetric assay was carried out in order to determine Cell viability. The cells were first seeded at 15000, 12000 and 10000 cells per well on 96-well plates for 24 h, 48 h and 72h assay respectively. After 24 h incubation, the cells were exposed to different concentration of free erlotinib (5, 10 and 20 μM dissolved in culture medium containing 0.1 % DMSO) and drug loaded nanoparticles (5, 10 and 20 μM suspended in culture medium). As the assay was conducted for 3 time intervals, culture medium in each well was replaced with 150 μl fresh medium and 50 μl MTT solution in PBS (2 mg/ ml) after 24, 48 and 72 hr incubation. The plates were then incubated for an additional 4 hours at 37°C following addition of MTT solution. The culture medium in each well was replaced by mixture of DMSO: Sorenson buffer (8:1) in order to dissolve purple formazan crystals. Absorbance was measured at 570 nm using microplate reader (Bio-Tek, USA). All tests were conducted in three replicate wells for each sample.

Results

Synthesis and characterization result of PCEC

Molar ratio of CL/PEG in synthesized triblock copolymers was 70, 280, 560 and 840. Synthesis of these copolymers was confirmed by FTIR as well as HNMR. An FTIR spectrum of synthesized PCEC triblock copolymer is shown in Figure 1. The absorption band at 1725 is related to stretching vibration of carbonyl group which confirms formation of copolymer (Ryu et al., 2001; Nguyen, 2010). Aliphatic CH stretching band of polyethylene oxide (PEO) and caprolactone were appeared at 2854 and 2923 cm-1 respectively. Intensity of these two absorption bands is dependent on the molar ratio of CL/ PEG in copolymers. Another absorption bands at 1111 and 1193 cm-1 are related to C-O-C stretching vibration. Two absorption bands were appeared at 1376 and 1460 cm-1 which related to methyl and methylene groups respectively. Figure 2 demonstrates HNMR of synthesized PCEC triblock copolymer. According to this spectrum, the singlet pick at 3.60 ppm is related to the methylene protons of the –CH2 CH2 O- units in PEG segment of copolymers. Also two triplets at 4.01and 2.26 ppm and two multiplets at 1.60and 1.35 ppm are related to methylene protons in PCL units. Therefore number of CL and PEG repeating unit in synthesized copolymers were determined from integral intensities of methylene protons at 4.03 and 3.61 ppm respectively. DSC thermograms of PEG and PCEC copolymers with different Cl/PEG molar ratio are shown in Figure 3. Melting peak at 61.64°C is observed in thermal curve of PEG. DSC thermogram of

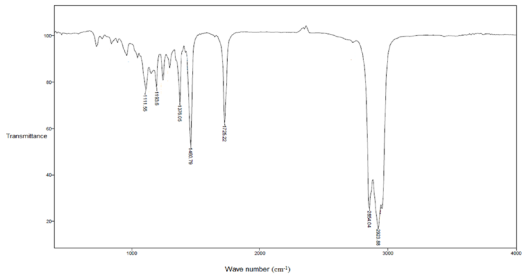

Figure 1. FTIR Spectra of Synthesized PCEC Triblock Copolymer (CL/PEG: 70)

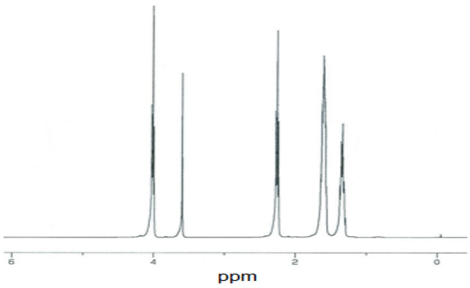

Figure 2. HNMR of synthesized PCEC triblock copolymer (CL/PEG: 280)

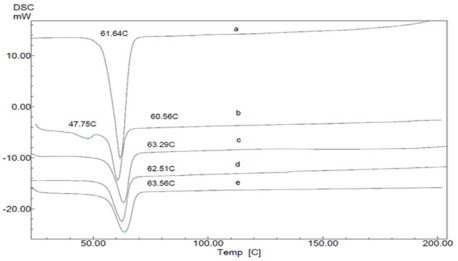

Figure 3. DSC thermogram of PEG (a) and PCEC Copolymers with Different CL/PEG Molar Ratio; 70(b), 280(c), 560(d) and 840 (e)

PCEC with molar ratio of 70, exhibits two endotherms at 47.75°C and 60.56°C which related to the melting of PEG and caprolactone blocks respectively. Melting point of PEG segment in this copolymer is lower than that of the PEG homopolymer. Melting point shift of PEG segment to lower temperature in PCEC copolymers was reported by Bogdanov et al. previously (Bogdanov et al., 1998). DSC curves of copolymers with higher CL/PEG molar ratio, show one endothermic peak which is related to melting point of CLblocks.

Results of preparation and characterization of nanoparticles

Synthesized PCEC triblock copolymers with different CL/PEG molar ratio (280, 560 and 840) were utilized for preparation of erlotinib nanoparticles. As PCEC copolymer with CL/PEG molar ratio of 70 was soluble in water, preparation of erlotinib nanoparticles using this copolymer was impossible. Compositions of prepared nanoparticles as well as their mean size, span value, zeta potential and encapsulation efficiency are presented in Table 1. Size of blank nanoparticles was 92 nm with a narrow distribution. Incorporation of drug into nanoparticles resulted in increased particle size. The particle size was decreased with decreasing the CL/PEG molar ratio in copolymers. Moreover diameter of particles is also depended on stabilizer concentration. One of the common parameters for determination of particle size distribution is span value which defines with the following equation.

Span = (D90% - D10%)/D50%

In which D90%, D10% and D50% are the diameters where 90%, 10% and 50% of particles are smaller than these sizes. A low value of span indicates a narrow size distribution and low polydispersity (Mondal et al., 2008). As indicated in table 1, span values of nanoparticles prepared with PCEC copolymer with CL/PEG molar ratio of 840 were high which determined their high polydispersity. However span values of nanoparticles with low CL/PEG molar ratio were low. For instance calculated span value for F5 (CL/PEG: 280, poloxamer concentration: 3%) was 0.70 which is below 1, indicating its narrow distribution. TEM image of PCEC nanoparticles indicates that nanoparticles were spherical in shape (Figure 4). Zeta potential of drug free nanoparticles was appeared to be -40.9 mV. Erlotinib loaded nanoparticles were negatively charged with zeta potential values ranging from -27.3 to -17.1 mV. It is obvious that negative charge of nanoparticles is attributed to PCL blocks of used copolymers.

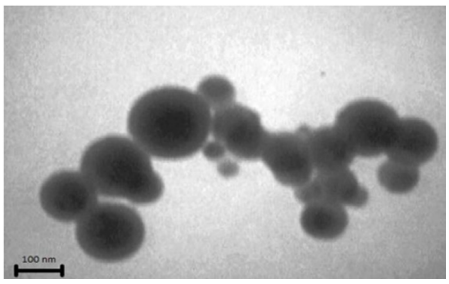

Figure 4. TEM Image of Nanoparticles (F5; CL/PEG: 280, Poloxamer Concentration: 3%)

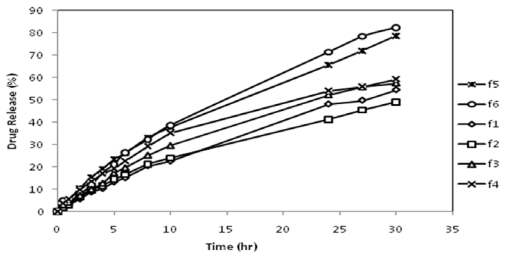

Figure 5. Release Profile of Erlotinib from Nanoparticles Prepared by (Copolymers with Different CL/PEG Ratio; various Surfactant Concentrations: F1: 840; 3%m, F2: 840; 1.5%, F3: 560; 3%, F4: 560; 1.5%, F5: 280; 3%, F6: 280; 1.5%)

Results of dissolution studies

Invitro release profile of erlotinib from nanoparticles in PBS containing 0.02% soy lecithin is shown in Figure 5. All formulations exhibited a sustained release pattern without any burst release. Amount of drug released from all formulations were in the range of 49-82% after 30 hours. From the release profiles, it can be found that erlotinib release rate from polymeric nanoparticles is declined with an increase of CL/PEG molar ratio of block copolymers. Nanoparticles formulated with lower CL/PEG molar ratio (280) exhibit highest release rate. Erlotinib release profiles

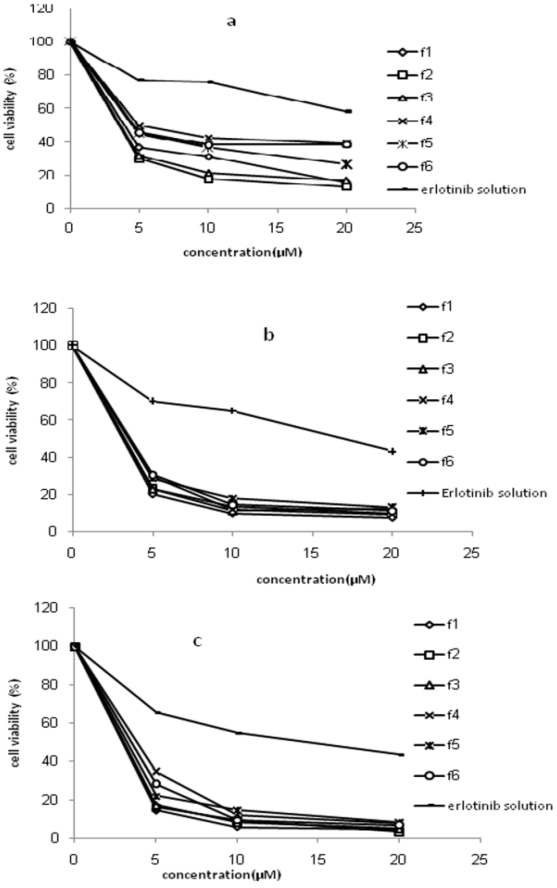

Figure 6. Viability of A549 Cells Incubated with Different Concentration of Erlotinib Solution and Nanoparticles After a: 24 hr b: 48 hr and c: 72 hr

Table 1. Mean Size, Span Value, Zeta Potential and Loading Efficiency (%) Of Nanoparticles

#	CL/PEG Molar ratio	Copolymer (mg)	Erlotinib (mg)	Poloxamer 188 (%)	Encapsulation efficiency(%)	Mean volume diameter (nm)	Zeta potential (mV)	Span value
F1	840	100	10	3	58.7	395	-24.4	4.5
F2	840	100	10	1.5	58.7	557	-17.1	14.7
F3	560	100	10	3	48.4	201	-20.4	1.4
F4	560	100	10	1.5	53.9	242	-26.6	1.1
F5	280	100	10	3	47.8	94	-27.3	0.7
F6	280	100	10	1.5	44.4	186	-26	1.1

from the nanoparticles formulated by copolymers with higher CL/PEG molar ratio (560, 840) exhibited more sustained release patterns.

Cytotoxicity results

The MTT colorimetric assay procedure was carried out in order to determine the cytotoxicity of drug loaded nanoparticles on A549 cell line. Invitro viability of A549 cells after incubation with different concentration of erlotinib solution and drug loaded nanoparticles are shown in Figure 6. Based on the obtained result, cell growth inhibition of erlotinib is dose and time dependent. Dependence of anti-proliferative activity on dose was more obvious following 72 hours of incubation. After 72 hours of exposure, the 50% inhibitory concentration (IC50) of erlotinib was 14.8 μM. It is evident that all drug loaded nanoparticles exhibited more anti proliferative activity in comparison to free erlotinib.

Discussion

PCEC triblock copolymers were focused in numerous investigations as drug carriers for cancer therapy. These amphiphilic copolymers could be used in various nanosized structures such as micelles, core-shell type nanoparticles and polymersomes in aqueous media. Hydrophobic core of PCEC nanoparticles are formed by PCL blocks which is surrounded by hydrophilic segment (Ryu et al., 2000; Zhang et al., 2011). Therefore erlotinib as a lipophilic drug can be entrapped within the core of these polymeric nanoparticles. The preparation method of nanosized structures from amphiphilic block copolymers are depended on the ratio of lipophilic/hydrophilic blocks. Copolymers with lower CL/PEG ratio can easily disperse in water and convert to micelles by self-assembling. On the other hand, copolymers with higher CL/PEG ratio are water-insoluble and can't self-assemble into nanoparticles upon direct dissolution. The common methods for preparation of nanoparticles with high CL/PEG copolymers are dialysis, emulsification and nanoprecipitation (Soppimath et al., 2001; Galindo-Rodriguez et al., 2004; Letchford and Burt, 2007). The solvent displacement method which is also called nanoprecipitation is a suitable method for incorporation of hydrophobic drugs to nanoparticles. Physicochemical properties of obtained polymeric nanoparticles depend on composition of used copolymers. Size of particles decreased with decreasing the CL/PEG molar ratio of copolymer. A similar observation has been reported previously, where particle size of polymeric nanoparticles obtained through nanoprecipitation method was decreased by decreasing the molecular weight or concentration of copolymers in acetone and also by increasing the surfactant concentration (Molpeceres et al., 1996; Ge et al., 2000; Ge et al., 2002). In nanoprecipitation method, formation of smaller nanodroplets during emulsification leads to increased specific surface area. Therefore diffusion of drug to external phase is enhanced which results in lower encapsulation efficiency (Sanchez et al., 1993; Fonseca et al., 2002).The direct effect of size on encapsulation efficiency of obtained particles is easily explained by this phenomenon with the exception of nanoparticles obtained using copolymers with CL/PEG molar ratio of 840 (F1 and F2). Although size of F2 nanoparticles was larger than F1 formulation, both of them exhibited the same encapsulation efficiency.

The prepared polymeric nanoparticles exhibited a sustained release pattern without any burst release. Erlotinib release rate from polymeric nanoparticles was decreased with an increased CL/PEG molar ratio. Erlotinib may physically incorporate in the lipophilic core of nanoparticles due to its lipophilic nature. Therefore by increasing CL/PEG molar ratio in copolymers, lipophilic segment of particles are raised and consequently release rate of erlotinib is declined because of its high binding affinity to the core of particles. As can be shown in Figure 5, amount of drug released from all formulations were in the range of 49-82% after 30 hours. Thus drug release rate could not be mainly controlled by polymer degradation because PCL degrades very slowly in the solution medium (Ryu et al., 2001; Ge et al., 2002). Therefore drug diffusion from polymeric nanospheres might be the main mechanism of release.

MTT colorimetric assay was performed in order to evaluate the cytotoxicity of free erlotinib and its nanoparticles. An inverse relationship between drug release rate during 24 hr and cytotoxicity (after 24 hr incubation) was observed. The slower release rate corresponds to higher cell toxicity. Although cytotoxicity induced by different drug loaded nanoparticles following 24 hours incubation was slightly varied, there was no significant differences in their cytotoxicity for longer incubation times (48 and 72 hrs).This might be attributed to the fact that the drug release from all formulations is almost complete at these time points (Figure 5). However, *in vitro* cytotoxicity of erlotinib, either as free drug or loaded in PCEC nanoparticles, was concentration and time dependent (Figure 6).

In conclusion, erlotinib loaded PCEC nanoparticles were prepared by solvent displacement method. Cytotoxicity studies demonstrated that incorporation of erlotinib in these nanocarriers enhances its antitumor

effect. Concerning suitable physicochemical properties such as low size and span values of nanoparticles prepared using PCEC with molar ratio of 280, it can be concluded that these nanoparticles might have the potential to be considered as novel delivery system for erlotinib.

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