Hacettepe University Journal of the Faculty of Pharmacy
Volume 33 / Number 1 / January 2013 / pp. 25-40

Formulation and *in Vitro* Evaluation of Paclitaxel Loaded Nanoparticles

Received : 11.07.2013
Revised : 19.12.2013
Accepted : 27.12.2013

Gözde Aygül*, Fırat Yerlikaya, Secil Caban**, İmran Vural**, Yılmaz Çapan**º**

Introduction

Cancer which forms a mass or a tumor by reproducing uncontrolled damaged cells in a particular tissue or an organ is one of the common causes of death worldwide (M.A. Knowles & P. Selby, 2005). Nanoparticulate systems used to deliver drug or biomolecules have been investigated for the purpose of cancer treatment (S. Feng & S. Chien, 2003). Polymeric nanoparticles have been frequently used in cancer treatment because of their advantages of increasing drug encapsulation efficiency, providing controlled release, reducing toxicity of the drug and being directed to the target.

Paclitaxel is a major anticancer chemotherapeutic agent, extracted from the bark of the Pacific yew tree (*Taxus brevifolia*) (S. Feng & G. Huang, 2001). It is widely used against various types of solid tumors (J.K. Jackson 2007). Paclitaxel stops cell division in the late mitotic phase by preventing microtubules destruction and inhibits the proliferation of the cells (R. Panchagnula, 1998, L. Vicari 2008). Taxol® containing

* Department of Nanotechnology and Nanomedicine, Faculty of Sciences, Hacettepe University, Ankara, Turkey
** Department of Pharmaceutical Technology2, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey
º Corresponding author: E-mail: ycapan@hacettepe.edu.tr

Cremophor® EL having an allergic effect is the commercial product of paclitaxel (A. Sparreboom 2005). The primary goal of formulation development for paclitaxel is to eliminate the Cremophor® EL thus reducing the toxic and allergic side effects, improving the formulation stability, bioavailability and providing controlled drug release.

Cancer treatment using paclitaxel is relying on the development of new delivery systems (J.D. Byrne 2008, B. Haley & E. Frenkel, 2008). Many nanoparticle systems which contain paclitaxel as the active ingredient have been investigated for the cancer treatment. The types of nanoparticles currently used in research for cancer therapeutic applications include dendrimers (T. Ooya 2003), liposomes (A. Sharma 1996), polymeric nanoparticles (S.S. Chakravarthi & D.H. Robinson, 2011), micelles (L. Zhang 2012), lipid emulsions (B.B. Lundberg 2003), niosomes (Z.S. Bayındır & N. Yuksel, 2009), and self-emulsifying drug delivery systems (SEDDS) (N. Gursoy 2003).

Nanoparticles which are solid colloidal particles ranging in size from 10 nm to 1000 nm have a great influence for targeted drug delivery systems (K.S. Soppimath 2001). Nanoparticles are made from biocompatible and biodegradable materials such as polymers, either natural (e.g., gelatin, albumin) or synthetic (e.g., polylactides, polyalkylcyanoacrylates), or solid lipids (D. Peer 2007). Poly(lactic-co-glycolic acid) (PLGA) is a hydrophobic copolymer which has many advantages to be used for drug delivery systems. Indeed, it is biocompatible, non-toxic, biodegradable and it has been approved by the United States Food and Drug Administration (FDA) in the use of drug delivery systems (J.M. Lu 2009).

Technological parameters such as polymer concentration and polymer composition have significant influences on characterization of nanoparticles. These parameters lead to variations in particle size and surface charge of nanoparticles which are very important parameters on their transmembranal passage and tissue targeting properties. Main objective of this study was to optimize the preparation of formulations by evaluating the influence of technological parameters on the physicochemical properties of PLGA nanoparticles.

For our goal, paclitaxel loaded nanoparticles were prepared in different concentrations of PLGA and polyvinyl alcohol (PVA) by emulsification-solvent diffusion method. The effects of PLGA and PVA concentrations on *in vitro* characteristics (particle size, zeta potential, encapsulation

efficiency, drug release) of nanoparticles were investigated. In addition, cell culture studies were conducted in order to evaluate the cytotoxicity of prepared paclitaxel loaded PLGA nanoparticles on Caco-2 cells.

Material and Methods

Material

Paclitaxel was purchased from Applichem, Germany. PLGA (50:50; Resomer® RG502H) was purchased from Boehringer-Ingelheim, Germany. PVA (M_w: 30-70 kDa), Dulbecco's modified Eagle's medium (DMEM), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and methanol were purchased from Sigma-Aldrich, USA. Dichloromethane, acetone and acetonitrile were purchased from Merck, Germany. Caco-2 cell culture was obtained from American Type Tissue Cell Culture Collection (ATTC), USA.

Methods

Preparation of paclitaxel loaded and blank PLGA nanoparticles

In this work, PLGA nanoparticles were prepared by emulsification-solvent diffusion method (K. Ozturk 2010). Briefly, PLGA (2%, 3%, 4%; *w*/*v*) and 1 mg of paclitaxel were dissolved in 10 ml acetone. The organic phase was emulsified with 20 ml of aqueous PVA solution (1%, 3%, 5%; *w*/*v*) under magnetic stirring. Then, the emulsion was homogenized by Ultra Turrax® (IKA, Germany) at 11000 rpm for 2 min and under magnetic stirring conditions. Then, 30 ml of deionized water was added into dispersion in 2 min to achieve the diffusion of organic phase to aqueous phase. Organic phase was, then, evaporated under room temperature by magnetic stirring for 24 h. The obtained nanosuspension was centrifuged at 13500 rpm for 30 min to collect nanoparticles. The supernatant containing the free drug was discarded and the pellet was freeze-dried for 48 h (Heto PowerDry PL3000, Denmark). Blank nanoparticles were prepared by an identical method (Fig. 1).

Effects of variations in polymer and emulsifier concentrations on the particle size distribution and surface charge of nanoparticles were assessed.

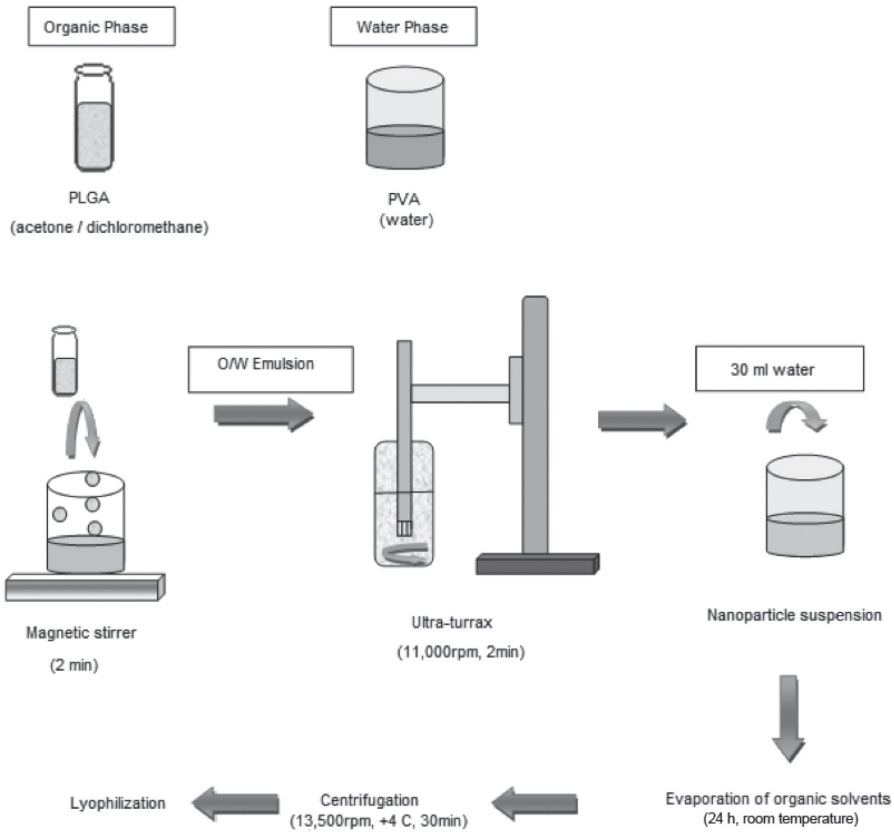

Figure 1
Schematic representation of the emulsification-solvent diffusion method used in this study.

Determination of paclitaxel content in the nanoparticles

The amount of entrapped paclitaxel in the nanoparticles was determined by HPLC. For HPLC analysis, an Agilent Technologies 1200 Series HPLC system with a quaternary pump, a degasser, an auto-sampler, a thermostatted column compartment, a variable wavelength detector and for data processing ChemStation B.02.01 software installed on a PC was used. The column was a Nucleodur C_{18} 250x4.6 mm i.d., 5 μm (Macherey Nagel, Germany). The flow rate of mobile phase was 1.0 ml/min at isocratic conditions. The injection volume was 10 μl, the column thermostat temperature was maintained at 40 °C and the detector wavelength was set at 227 nm. The mobile phase was comprised of ultrapure water and

acetonitrile (45:55, *v*/*v*) (C. Jin 2009). The calibration curve for the quantification of paclitaxel was linear over the range of standard concentration of paclitaxel at 1-20 μg/mL with a correlation coefficient of 0.9999. The developed method was validated with respect to its specificity, accuracy, precision, and linearity (ICH. Validation of Analytical Procedures: Text and Methodology, 2005).

For the assay of paclitaxel in the prepared nanoparticles a specific amount of lyophilized nanoparticles was dissolved in methanol. The mixture was then sonicated for 15 min. The solution was filtered through 0.22 μm syringe filter and then analyzed by HPLC. The drug encapsulation efficiency was expressed as the percentage of the drug amount found in the nanoparticles to the total drug amount added in the process.

Particle size distribution and zeta potential

Average particle, polydispersity index and zeta potential were determined by photon correlation spectroscopy using a Zetasizer Nanoseries ZS (Malvern Instruments, UK). The analysis was performed at a scattering angle of 90 ° at a temperature of 25 °C using samples appropriately diluted with ultrapure water.

In vitro release studies

For the in vitro release studies, a solution of phosphate-buffered saline (PBS, pH 7.4) containing 0.05% (*w*/*v*) sodium lauryl sulfate was used for release medium (J.S. Chawla & M.M. Amiji, 2002). 10 mg of nanoparticles was placed into polypropylene tubes containing 1 ml of release medium and the tubes were incubated in an orbital shaker water bath (Memmert, Germany). The water bath was maintained at 37 °C and shaken horizontally at 50 rpm (B. Mukherjee 2008). At designated time points, the tubes were taken out from the water bath and were centrifuged at 13500 rpm for 15 min. 0.5 ml of supernatant was collected from each tube for HPLC analysis and 0.5 ml of fresh release medium was added and particles were resuspended.

Paclitaxel drug loading and association efficiency values were calculated according to the following equations: % Drug Loading = 100 x (total paclitaxel amount-free paclitaxel amount)/NP weight and % Encapsulation Efficiency=100 x (total paclitaxel amount-free paclitaxel amount)/ total paclitaxel amount.

In vitro cytotoxicity

Cytotoxicity of the formulations on Caco-2 cells was assessed using the MTT method. MTT assay was performed to evaluate cell viability. The MTT assay is a colorimetric measurement of MTT reduction to a blue formazan product by mitochondrial dehydrogenases of viable cells (P.R. Twentyman & M. Luscombe, 1987).

Caco-2 cells were seeded in 96-well plates. For the cytotoxicity experiments the cells were further incubated with paclitaxel solution, a drug free and a drug loaded formulation (containing 250 nM paclitaxel, 2% (*w*/*v*) PLGA and 3% (*w*/*v*) PVA) and the DMEM as a control group for 24 and 48 hours at 37 °C and 5% CO_2. After incubation the culture medium was removed. 20 µl of MTT solution was added to each well, followed by incubation at 37 °C for 4 h to allow formazan formation. The medium and MTT were removed and 200 µl of dimethylsulfoxide was added to dissolve the formazan crystals. Absorbance at 570 nm was measured using an ELISA plate reader (ASYS-UVM 304, Austria).

Results and Discussion

Nanoparticle preparation and characterization

Aiming at determination of the influences of the technological parameters on developed nanoparticles, the physicochemical properties of the nanoparticles, in terms of their particle size distribution and surface charge were evaluated.

Blank and paclitaxel loaded PLGA nanoparticles were prepared by emulsion-solvent diffusion method. For this, PLGA was dissolved in acetone or dichloromethane (R. Dinarvand 2011). In our study, we used both organic solvents for blank nanoparticle preparations. We investigated the effects of the type of organic phase solvents on the average particle size and polydispersity index (PDI) of PLGA nanoparticles.

Table I and Table II show effects of the organic solvent on the nanoparticle size and PDI. The smallest nanoparticles (91±0.9 nm) were obtained with 2% PLGA (*w*/*v*) concentration in acetone and 1% PVA (*w*/*v*) in aqueous phase which were prepared by emulsification-solvent diffusion technique.

Acetone is completely miscible with water in all proportions and is a good solvent for PLGA. Dichloromethane is immiscible with water and is an excellent solvent for PLGA (K.C. Songa 2006). Small particles were obtained using partially water-soluble solvents such as acetone while large particles were obtained using water immiscible solvent such as dichloromethane.

Particle size distribution showed that the obtained nanoparticles were monodisperse systems. PDI of blank PLGA nanoparticles prepared with dichloromethane was found between 0.22 and 0.35, and PDI of nanoparticles prepared with acetone was found between 0.02 and 0.07. It can be seen that particles prepared with acetone have narrower size distribution than dichloromethane (Table I and II).

Because of immiscibility with water of dichloromethane as the organic solvent, formulation resulted in aggregation. On the other hand, as acetone is completely miscible with water, stable emulsions between organic and aqueous phases were formed.

Zeta potential of nanoparticles was found to be negative and it ranged from -19.2 mV to -12.7 mV (Table I and II). The presence of the terminal carboxylic acid groups on the surface of PLGA lead to a negative zeta potential of nanoparticles (M.F. Zambaux 1998).

TABLE I
Particle size distribution and zeta potential of blank PLGA nanoparticles prepared with dichloromethane (n=3) (mean ± standard deviation).

PVA Concentration (%)	Average particle size (nm)	PDI	Zeta potential (mV)
1	140±5	0.22	-12.7±1.4
3	230±6	0.35	-17.3±0.7
5	173±7	0.23	-19.0±0.7

TABLE II
Particle size distribution and zeta potential of blank PLGA nanoparticles prepared with acetone (n=3) (mean ± standard deviation).

PVA Concentration (%)	Average particle size (nm)	PDI	Zeta potential (mV)
1	91±0.9	0.07	-14.1±2.0
3	111±2.0	0.06	-19.2±0.7
5	104±1.7	0.02	-18.2±0.2

In our study, blank formulations with smaller particle size and PDI were obtained with acetone. Therefore, paclitaxel loaded PLGA nanoparticle formulations were prepeared by using acetone in organic phase. In these formulations we used different PLGA and PVA concentrations. The average particle size and PDI of all paclitaxel loaded nanoparticles were determined and given in Table III.

According to the results obtained, particle size of paclitaxel loaded nanoparticles is slightly larger than blank nanoparticles. The size of nanoparticles was found in the range of 190-235 nm. In order to investigate the effects of polymer concentration on the formulation properties, different concentrations of polymer in the oil phase were used to fabricate the nanoparticles. The nanoparticle size increased with increasing concentration of the polymer. It was shown that the size of nanoparticles was smaller when fabricated with 2% (*w*/*v*) PLGA as polymer. When PLGA concentration was increased, particle size was increased as well. This was probably caused by the increasing viscosity and hence resulting poor dispersibility of the PLGA solution into the aqueous phase (R.M. Mainardes & R.C. Evangelista, 2005).

PDI of paclitaxel loaded PLGA nanoparticle formulations was found between 0.06 and 0.20 (Table III). All formulations showed uniform particle size distribution.

In our study, we also investigated the influence of different PVA concentrations in the external aqueous phase. As shown in Table 3, there was a decrease in particle size when the PVA concentration in the external aqueous phase was increased from 1% to 5% (*w*/*v*). In the emulsification process stabilizer can avoid the coalescence of globules. Therefore, a high concentration of droplet stabilizer leads to a reduced size of the nanoparticles produced (H. Murakami 1999).

Nanoparticles had a negative surface charge. Zeta potential is an important particle characteristic as it is commonly an index of the stability of the nanoparticles. Zeta potential and physical stability of PLGA nanoparticles were observed to increase with increasing PVA concentration. Zeta potential values of the prepared paclitaxel loaded PLGA nanoparticles vary between -13.9 and -21.9 mV (Table III).

Paclitaxel content in the nanoparticles

An HPLC method with UV detection was developed for the assay of paclitaxel in PLGA nanoparticles and validated according to the ICH

TABLE III
Particle size and zeta potential of paclitaxel loaded nanoparticle formulations containing various amounts of PVA in aqueous phase and PLGA in organic phase (n=3) (mean ± standard deviation).

PLGA Concentration (%, w/v)	PVA Concentration (%, w/v)	Average particle size (nm)	PDI	Zeta potential (mV)
2	1	219±8	0.20	-13.9±1.4
	3	191±1	0.06	-21.9±0.4
	5	190±1	0.08	-16.1±1.6
3	1	195±10	0.09	-16.4±0.2
	3	235±6	0.15	-16.5±1.7
	5	223±5	0.13	-17.2±0.2
4	1	214±4	0.12	-14.4±0.4
	3	234±5	0.14	-15.0±0.3
	5	224±3	0.17	-16.6±0.5

recommendations. As no interference was observed between paclitaxel peak and the peaks from matrix the method was found specific to paclitaxel. In addition; the coefficient of determination of the regression line was found to be higher than 0.999, indicating method's linearity over the working concentration range (1-20 μg/ml); the intra- and inter-assay coefficient of variation values were both found to be lower than 2%, showing method's repeatability and intermediate precision; and the percent recovery values were found to be between 98-102% which confirmed method's accuracy.

Table IV shows encapsulation efficiency of the paclitaxel loaded PLGA nanoparticle formulations. The measured encapsulation efficiency ranged from 53% to as much as 89%. The encapsulation efficiency was highly dependent on the concentration of PLGA since it increased as the polymer concentration increased in the oil phase and PVA concentration decreased in the aqueous phase. It was observed that increasing PVA concentrations resulted in decreased levels of encapsulation. Because the solubility of paclitaxel in aqueous phase increases with higher surfactant concentrations the diffusion of paclitaxel from inner phase to outer phase also increases (Q. Yang, 2000). On the contrary, drug loading values decreased because total nanoparticle weight increased when polymer concentration increased.

TABLE IV
Results of paclitaxel entrapment into PLGA nanoparticles (n=3).

PLGA Concentration (%, w/v)	PVA Concentration (%, w/v)	Encapsulation efficiency (%)	Drug loading (%)
2	1	82.6	52.5
	3	53.0	41.7
	5	62.7	71.8
3	1	86.6	37.9
	3	79.6	33.2
	5	71.4	19.0
4	1	89.0	28.9
	3	85.4	24.5
	5	68.6	23.5

In vitro release of paclitaxel from nanoparticles

The release behavior of paclitaxel from the nanoparticle formulations showed a biphasic pattern: fast release rate in the first day followed by a slow uniform release afterwards. The burst release of paclitaxel may be due to the dissolution and diffusion of the drug that was poorly entrapped in the polymer matrix or weakly bound or adsorbed drug to the surface of nanoparticles, while the slower and continuous release may be attributed to the diffusion of the drug localized in the PLGA core of the nanoparticles. The particle size is important for the release profile of the drug from the particles, as smaller particles have a larger surface area. This might result in a high burst release of the drug and a shorter period of sustained release (A. Budhian 2005). Because of the hydrophobic interactions between paclitaxel and PLGA, the release profiles published in the literature are relatively slow and the cumulative release is usually between 5% and 35%. Formulations having PLGA with acid end-groups exhibits faster release profiles. Acid-terminated PLGA has free carboxyl groups and therefore the polymer can provide more swelling due to water uptake comparing with ester-terminated PLGA which is more hydrophobic and thus acid-terminated PLGA degrades more rapidly. In addition, using low-molecular-weight PLGA in

formulations contributes to a faster degradation as it is easier forming of the pores due to less hydrophobicity of the polymer. Moreover, paclitaxel has low water solubility, thus the diffusion is not the primary release mechanism. Hence, the release kinetics of paclitaxel from nanoparticles might not be explained by diffusion but can be linked to self-erosion of the matrix (F. Yerlikaya, 2013).

Fig. 2 shows paclitaxel release from the nanoparticles. The cumulative percentages of the released paclitaxel from the experimental formulations were plotted against time, it was found that about 80% paclitaxel was released in 7 days in case of the formulation 4% PLGA-3% PVA, whereas, 2% PLGA-%5 PVA had a release of about 36% in the same time period. As can be observed, nanoparticle formulations with a concentration 4% PLGA-3% PVA showed the fastest release profile as it had a larger average particle size.

Cytotoxicity

Blank PLGA nanoparticles and paclitaxel loaded nanoparticles were tested for their cytotoxic effects on Caco-2 cells. To evaluate cytotoxicity, PLGA nanoparticles were added to Caco-2 cells and cell viability after 24 and 48 h was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As expected, in the tested control group showed no toxicity on Caco-2 cell line. Blank nanoparticles did not cause significant cytotoxicity against cell lines. Results for blank nanoparticle and control group were showed similar non-toxic effect. On the other hand, paclitaxel loaded nanoparticles showed cytotoxicity against tested cell lines. The comparison between paclitaxel loaded PLGA nanoparticles and paclitaxel solution showed that paclitaxel loaded PLGA nanoparticles significantly reduced cell viability than paclitaxel solution (Fig. 3).

Conclusion

In this study, it was shown that increasing PVA concentration in aqueous phase and decreasing PLGA concentration in organic phase decreased the mean particle size of nanoparticles. PDI of paclitaxel loaded nanoparticles were found to be lower than 0.2 indicating narrow size

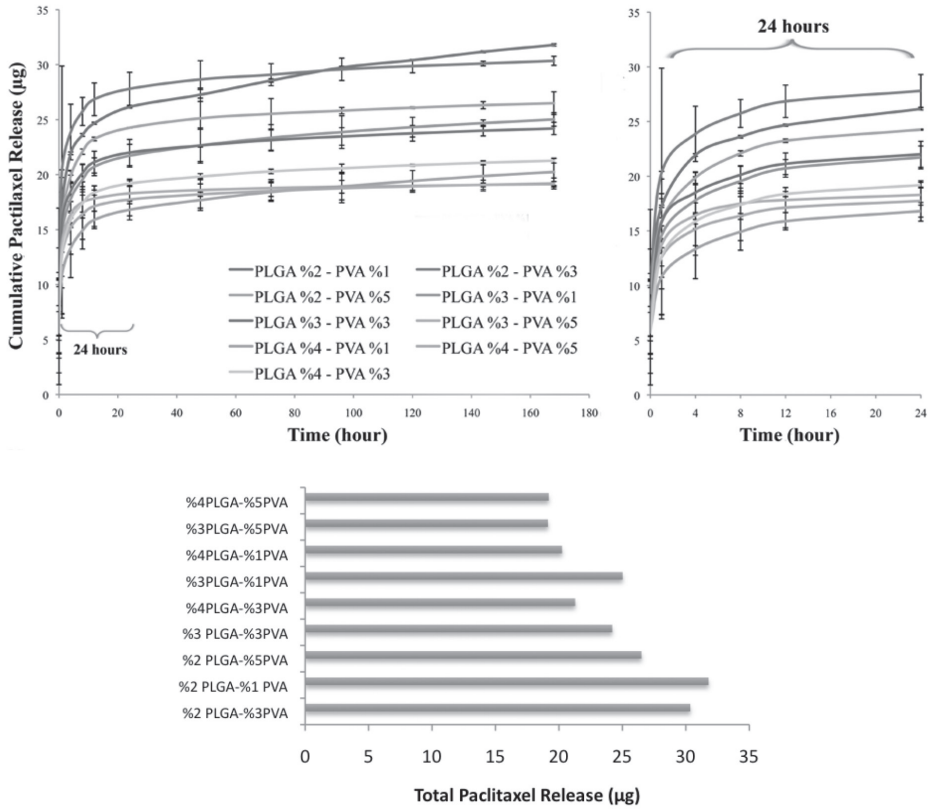

Figure 2
In vitro drug release profiles of paclitaxel loaded PLGA nanoparticle formulations *(n=3)* and total amount of paclitaxel released from PLGA nanoparticles at the end of the release study.

distributions. There was no significant difference in surface charges of nanoparticles which were prepared with different polymer and emulsifier concentrations. Within a certain range of particle size, encapsulation efficiency decreased and drug release rate increased with reducing particle sizes. Cytotoxicity tests showed that the paclitaxel loaded PLGA nanoparticles had higher cell toxicity than the blank nanoparticles. Paclitaxel loaded PLGA nanoparticles and paclitaxel solution showed similar cell toxicity effects on Caco-2 cell lines. In addition, a reversed-phase HPLC method using UV detection for the determination of paclitaxel was developed. The method was found to be specific, linear, accurate, precise and

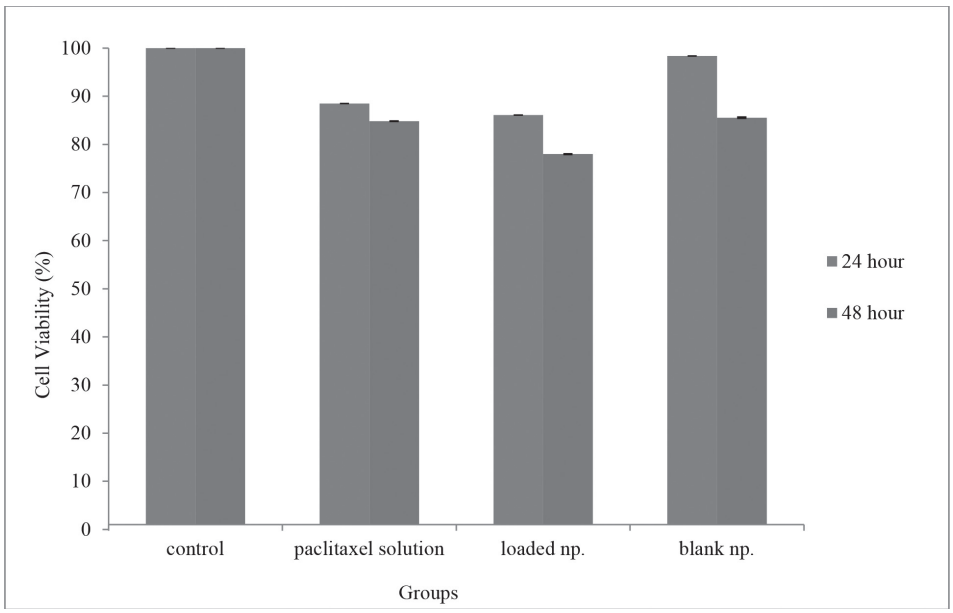

Figure 3
Cytotoxic effects on Caco-2 cells of paclitaxel formulations. *(n=3).*

sensitive to determine paclitaxel content in as well as released from PLGA nanoparticles. The method can be used in new drug delivery systems where paclitaxel is the active substance.

Summary

In this study, paclitaxel loaded PLGA nanoparticles were prepared using emulsification-solvent diffusion method and the influences of polymer and emulsifier concentrations on particle size, zeta potential, encapsulation efficiency and *in vitro* release rate of the paclitaxel loaded PLGA nanoparticles were evaluated. It was observed that average particle size of the prepared nanoparticles were between 190 and 235 nm. The encapsulation efficiency of paclitaxel into the nanoparticles was between 53-89% and the release profiles of the nanoparticles exhibited an initial burst effect followed by a controlled release for 7 days. *In vitro* cytotoxicity

studies showed that drug loaded nanoparticle formulation was more cytotoxic than the blank nanoparticle formulation. According to the results of the studies performed, it can be concluded that prepared nanoparticle formulations may be a considerable approach for paclitaxel delivery.

Keywords: Paclitaxel, PLGA, Formulation, Nanoparticles

Özet

Paklitaksel yüklü nanopartiküllerin formülasyonu ve *in vitro* incelenmesi

Bu çalışmada, paklitaksel yüklü PLGA nanopartikülleri emülsifikasyon-solvent difüzyon metodu kullanılarak hazırlanmış ve polimer ve yüzey aktif madde konsantrasyonunun paklitaksel yüklenmiş PLGA nanopartiküllerini partikül büyüklüğü, zeta potansiyel, enkapsülasyon etkinliği ve *in vitro* salım oranı üzerine etkisi incelenmiştir. Hazırlanan nanopartiküllerin ortalama partikül büyüklüğünün 190 ve 235 nm arasında olduğu gözlemlenmiştir. Paklitakselin nanopartiküllerin içine ankapsülasyon etkinliği %53-89 arasında bulunmuş olup nanopartiküllerin salım profillerinde başlangıçta patlama etkisini takiben 7 günlük kontrollü salım gözlemlenmiştir. *In vitro* sitotoksisite çalışmaları ilaç yüklü nanopartikül formülasyonlarının yüklenmemiş olanlara kıyasla daha sitotoksik olduğunu göstermiştir. Yapılan çalışmalar sonucunda, hazırlanan formülasyonun paklitaksel taşınmasında kullanılabileceği bulunmuştur.

Anahtar kelimeler: Paklitaksel, PLGA, Formülasyon, Nanopartiküller

REFERENCES

1. Bayındır ZS, Yuksel N. Characterization of niosomes prepared with various nonionic surfactants for paclitaxel oral delivery, J Pharm Sci, 99,2049-60. (2009)
2. Budhian A, Winey KI, Siegel SJ. Production of haloperidol loaded PLGA nanoparticles for extended controlled drug release of haloperidol, J Microencapsulation, 22,773-85. (2005)
3. Byrne JD, Betancourt T, Brannon-Peppas L.: Active targeting schemes for nanoparticle systems in cancer therapeutics, Adv Drug Deliv Rev, 60,1615-26. (2008)
4. Chakravarthi SS, Robinson DH. Enhanced cellular association of paclitaxel delivered in chitosan-PLGA particles, Int J Pharm, 409,111-20. (2011)

5. Chawla JS, Amiji MM. Biodegradable poly(o-caprolactone) nanoparticles for tumor targeted delivery of tamoxifen, Int J Pharm, 249,127-38. (2002)
6. Dinarvand R, Sepehri N, Manoochehri S, Rouhani H, Atyabi F. Polylactide-co-glycolide nanoparticles for controlled delivery of anticancer agents, Int J Nanomedicine, 6,877-95. (2011)
7. Feng S, Chien S. Chemotherapeutic engineering: application and further development of chemical engineering principles for chemotherapy of cancer and other diseases, Chem Eng Sci, 4087 – 114. (2003)
8. Feng S, Huang G. Effects of emulsifiers on the controlled release of paclitaxel (Taxol) from nanospheres of biodegradable polymers, J Control Release, 71,53-69. (2001)
9. Gursoy N, Garrigue JS, Razafindratsita A, Lambert G, Benita S. Excipient effects on in vitro cytotoxicity of a novel paclitaxel self-emulsifying drug delivery system, J Pharm Sci, 92,2411-8. (2003)
10. Haley B, Frenkel E. Nanoparticles for drug delivery in cancer treatment, Urol Oncol, 26,57-64. (2008)
11. ICH. Q2(R1) Validation of Analytical Procedures: Text and Methodology. (2005)
12. Jackson JK, Hung T, Letchford K, Burt HM. The characterization of paclitaxel-loaded microspheres manufactured from blends of poly(lactic-co-glycolic acid) (PLGA) and low molecular weight diblock copolymers, Int J Pharm, 342,6-17. (2007)
13. Jin C, Bai L, Wu H, Song W, Guo G, Dou K. Cytotoxicity of paclitaxel incorporated in PLGA nanoparticles on hypoxic human tumor cells, Pharm Res, 26,1776-84. (2009)
14. Knowles MA, Selby P, New York: Oxford University Press. (2005),
15. Lu JM, Wang X, Marin-Muller C, Wang H, Lin PH, et al. Current advances in research and clinical applications of PLGA-based nanotechnology, Expert Rev Mol Diagn, 9,325-41. (2009)
16. Lundberg BB, Risovic V, Ramaswamy M, Wasan KM. A lipophilic paclitaxel derivative incorporated in a lipid emulsion for parenteral administration, J Control Release, 86,93-100. (2003)
17. Mainardes RM, Evangelista RC. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution, Int J Pharm, 290,137-44. (2005)
18. Mukherjee B, Santra K, Pattnaik G, Ghosh S. Preparation, characterization and in-vitro evaluation of sustained release protein-loaded nanoparticles based on biodegradable polymers, Int J Nanomedicine, 3,487-96. (2008)
19. Murakami H, Kobayashi M, Takeuchi H, Kawashima Y. Preparation of poly(DL-lactide-co-glycolide) nanoparticles by modified spontaneous emulsification solvent diffusion method, Int J Pharm, 187,143-52. (1999)
20. Ooya T, Lee J, Park K.: Effects of ethylene glycol-based graft, star-shaped, and dendritic polymers on solubilization and controlled release of paclitaxel, J Control Release, 93,121-7. (2003)
21. Ozturk K, Caban S, Kozlu S, Kadayifci E, Yerlikaya F, Capan Y. The influence of technological parameters on the physicochemical properties of blank PLGA nanoparticles, Pharmazie, 65,665-9. (2010)
22. Panchagnula R. Pharmaceutical aspects of paclitaxel, Int J Pharm, 1-15. (1998)
23. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy, Nature Nanotech, 2,751 - 60. (2007)
24. Sharma A, Sharma US, Straubinger RM. Paclitaxel-liposomes for intracavitar therapy of intraperitoneal P388 leukemia, Cancer Lett, 107,265-72. (1996)

25. Songa KC, Lee HS, Chounga Y, In Choa K, Ahn Y, Choi EJ. The effect of type of organic phase solvents on the particle size of poly(d,l-lactide-co-glycolide) nanoparticles, Colloid Surface A, 276,162–7. (2006)

26. Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE. Biodegradable polymeric nanoparticles as drug delivery devices, J Control Release, 70,1-20. (2001)

27. Sparreboom A, Scripture CD, Trieu V, Williams PJ, De T, et al. Comparative preclinical and clinical pharmacokinetics of a cremophor-free, nanoparticle albumin-bound paclitaxel (ABI-007) and paclitaxel formulated in Cremophor (Taxol), Clin Cancer Res, 11,4136-43. (2005)

28. Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity, Br J Cancer, 56,279-85. (1987)

29. Vicari L, Musumeci T, Giannone I, Adamo L, Conticello C, et al. Paclitaxel loading in PLGA nanospheres affected the in vitro drug cell accumulation and antiproliferative activity, BMC Cancer, 8,212. (2008)

30. Yang Q, Owusu-Ababio G. Biodegradable progesteronemicrosphere delivery system for osteoporosis therapy, Drug Dev Ind Pharm, 26, 61–70. (2000)

31. Yerlikaya F, Ozgen A, Vural I, Guven O, Karaagaoglu E, Khan MA, Capan Y. Development and evaluation of paclitaxel nanoparticles using a quality-by-design approach, J Pharm Sci., 102, 3748-61. (2013)

32. Zambaux MF, Bonneaux F, Gref R, Maincent P, Dellacherie E, et al. Influence of experimental parameters on the characteristics of poly(lactic acid) nanoparticles prepared by a double emulsion method, J Control Release, 50,31-40. (1998)

33. Zhang L, He Y, Ma G, Song C, Sun H. Paclitaxel-loaded polymeric micelles based on poly(varepsilon-caprolactone)-poly(ethylene glycol)-poly(varepsilon-caprolactone) triblock copolymers: in vitro and in vivo evaluation, Nanomedicine, 8,925-34. (2012)