Biodegradable Nanoparticles Containing Doxorubicin-PLGA Conjugate for Sustained Release

Hyuk Sang Yoo,[1] Jong Eun Oh,[2] Keun Hyeung Lee,[2] and Tae Gwan Park[1,3]

Received December 15, 1998; accepted March 13, 1999

Purpose. Doxorubicin was chemically conjugated to a terminal end group of poly(D,L-lactic-co-glycolic acid) [PLGA] and the doxorubicin-PLGA conjugate was formulated into nanoparticles to sustain the release of doxorubicin.

Methods. A hydroxyl terminal group of PLGA was activated by p-nitrophenyl chloroformate and reacted with a primary amine group of doxorubicin for conjugation. The conjugates were fabricated into *ca.* 300 nm size nanoparticles by a spontaneous emulsion-solvent diffusion method. The amount of released doxorubicin and its PLGA oligomer conjugates was quantitated as a function of time. The cytotoxicity of the released species was determined using a HepG2 cell line.

Results. Loading efficiency and loading percentage of doxorubicin-PLGA conjugate within the nanoparticles were 96.6% and 3.45 (w/w) %, respectively while those for unconjugated doxorubicin were 6.7% and 0.26 (w/w) %, respectively. Both formulation parameters increased dramatically due to the hydrophobically modified doxorubicin by the conjugation of PLGA. The nanoparticles consisting of the conjugate exhibited sustained release over 25 days, whereas those containing unconjugated free doxorubicin showed rapid doxorubicin release in 5 days. A mixture of doxorubicin and its PLGA oligomer conjugates released from the nanoparticles had comparable IC_{50} value in a HepG2 cell line compared to that of free doxorubicin. Sustained drug release was attributed to the chemical degradation of conjugated PLGA backbone, which permitted water solubilization and subsequent release of doxorubicin conjugated PLGA oligomers into the medium.

Conclusions. The conjugation approach of doxorubicin to PLGA was potentially useful for nanoparticle formulations that require high drug loading and sustained release. The doxorubicin-PLGA oligomer conjugate released in the medium demonstrated a slightly lower cytotoxic activity than free doxorubicin in a HepG2 cell line.

KEY WORDS: poly(lactic-co-glycolic acid); doxorubicin; conjugation; nanoparticles; sustained release.

INTRODUCTION

Recently, anti-cancer drugs have been chemically conjugated to various polymers for the purpose of its efficient passive targeting to solid tumors (1–3). The "enhanced permeation and retention (EPR)" effect on the site of tumor capillaries plays a critical role in accumulating the polymer conjugates in the solid tumors, while minimizing the glomerular excretion rate (4,5). Water soluble polymer conjugates based on poly(N-(2-hydroxypropyl)methacrylamide) has been extensively studied and are now under clinical trials (6). Another promising approach is to conjugate doxorubicin to an amphiphilic block copolymer composed of polyethyleneglycol (PEG) and poly(α,β-aspartic acid), which leads to a polymeric micelle structure (7). Besides, in the above examples, doxorubicin has been physically adsorbed onto and/or encapsulated within nondegradable and biodegradable nanoparticles (8,9), protein nanoparticles (10), and liposomes (11,12). The above doxorubicin formulations intend to achieve passive targeting of doxorubicin loaded particulates to the tumor site.

We have previously reported a hydrophilic model drug, an amino acid derivative, could be chemically conjugated to a terminal end group of poly(D,L-lactic-co-glycolic acid) [PLGA] to increase its hydrophobicity and, the resultant drug-PLGA conjugates could be formulated into microspheres by a single oil-in-water emulsion technique (13). The loading efficiency was almost 100% due to the covalent linkage of the target compound to PLGA. Additionally, the release profiles of drug and its PLGA oligomer conjugates from the microspheres exhibited a near zero order of kinetic behavior over an extended period, while the microspheres containing unconjugated drug demonstrated an initial rapid release in the early stage of incubation. This novel formulation strategy is based on the hypothesis that drugs can not be released from microspheres until the PLGA polymer chain gradually degrades and reaches a critical molecular weight at which the water solubility of drug-PLGA oligomer conjugate is sufficiently gained. Thus, the drug release rate is expected to solely depend on the chemical degradation rate of conjugated PLGA chain, which permits the controlled liberation of the conjugated drug in a mixture of water soluble drug-PLGA oligomer conjugates. The conjugation approach is expected to be particularly suitable for the formulation of nanoparticles which normally require high drug payload and a sustained release property.

In this study, doxorubicin was conjugated to PLGA by first activating a hydroxyl terminal group of PLGA by p-nitrophenyl chloroformate and then by reacting it to a primary amine group of doxorubicin. The doxorubicin-PLGA conjugate was directly formulated into nanoparticles using a spontaneous emulsion-solvent diffusion method (14). Doxorubicin loading efficiency, loading amount, and release characteristics of the formulated nanoparticles were investigated in comparison to those of the nanoparticles containing free doxorubicin. Cytotoxicity of the released doxorubicin-PLGA oligomer conjugates was determined using free doxorubicin as a positive control. The major objective of this study is to prepare biodegradable nanoparticles having a high doxorubicin payload, which hold an additional sustained release capability at the site of passive targeting in solid tumors.

MATERIALS AND METHODS

Materials

Poly(D,L-lactic-co-glycolic acid) having lactic/glycolic molar ratio of 50/50 was obtained from Wako Chemicals (Japan). [PLGA5005]. The weight average molecular weight was 8020 as determined from gel permeation chromatography. This polymer has free hydroxyl and carboxylic groups at its terminal ends. Doxorubicin, p-nitrophenyl chloroformate, and

[1] Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, South Korea.

[2] Mogam Biotechnology Research Institute, 341 Pojung-ri, Koosung-myun, Yongin, Kyunggido 449-910, South Korea.

[3] To whom correspondence should be addressed. (e-mail: tgpark@sorak.kaist.ac.kr)

0724-8741/99/0700-1114$16.00/0

3-(4,5-dimethylthiaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. All other chemicals were analytical grade.

Methods

Conjugation of Doxorubicin to PLGA

One gram of PLGA dissolved in 10 ml of methylene chloride was activated by 72 mg of p-nitrophenyl chloroformate by adding 47 mg of pyridine (PLGA/p-nitrophenyl chloroformate/pyridine stoichiometric molar ratio: 1/2.8/4.8) in a dropwise manner into the solution at 0°C. The reaction was carried out for 3 hr at room temperature under nitrogen atmosphere. The reaction progress was monitored by gel permeation chromatography (GPC) with dual UV detection at 230 nm and 260 nm for the ester group in the PLGA backbone and p-nitrophenyl group in the activated PLGA end group, respectively. The resultant solution was diluted by methylene chloride and washed with 0.1% HCl and brine solution. The organic phase was separated, dried on sodium sulfate, and then dried under vacuum (yield: 80%). The activated PLGA (0.1 g) dissolved in 3 ml of dimethylformamide (DMF) was reacted with 6.3 mg of doxorubicin in the presence of 5 mg of triethylamine for 24 hr at room temperature under nitrogen atmosphere (stoichiometric molar ratio of activated PLGA/doxorubicin/triethylamine: 1/0.8/4). The progress of doxorubicin conjugation to activated PLGA was monitored by GPC by an UV-Vis dual wavelength at 230 nm and 480 nm which detected the fraction of PLGA and doxombicin conjugated PLGA, respectively. The precipitated product by the addition of cold diethyl ether was filtered and dried. The yield of conjugation reaction was 58%. The extent of doxorubicin conjugation to PLGA was determined by dissolving the conjugate in dimethylsulfoxide (DMSO), and then its absorbance was measured at 480 nm. A series of doxorubicin with different concentrations in DMSO were used as calibration standards.

Gel Permeation Chromatography (GPC) of Doxorubicin-PLGA Conjugates

The synthesized conjugates were characterized by GPC using Gilson 306 pump with UV-Vis detector. The GPC column was Shodex K-803 (300 × 7.8 mm, Phenomenex, USA) and tetrahydrofuran was used as a mobile phase with a flow rate of I ml/min. Molecular weight of the conjugate was calculated using a series of polystyrene standards (Mr: 114,200, 44,000, 13,700, and 3,700).

Preparation and Characterization of Nanoparticles

Two types of nanoparticles containing doxorubicin-PLGA conjugate and free doxorubicin were prepared by a spontaneous emulsion solvent diffusion method. One hundred mg of PLGA-doxorubicin conjugate dissolved in 10 ml of acetone was slowly added to 100 ml of deionized water containing 1% (w/v) Pluronic F-127 under vigorous stirring conditions. For the encapsulation of free doxorubicin into nanoparticles, 95 mg of PLGA and 5 mg of doxorubicin were co-dissolved in acetone and then used. The nanoparticles formed in the aqueous solution were collected by ultracentrifugation (Beckman, USA) at 15,000 rpm for 1 hr and resuspended in phosphate buffered saline (PBS) solution. The resuspended nanoparticles were stored under frozen conditions (−20°C) until use. The loading a mount of doxorubicin within nanoparticles was determined by a spectroscopic method. A known amount of freeze dried nanoparticles was completely dissolved in dimethylsulfoxide (DMSO) and then the absorbance was measured at 480 nm according to the aforementioned method. Encapsulation efficiency was calculated based on the percent ratio of the amount of doxorubicin incorporated into nanoparticles to the initial amount used. Size distribution was measured by using a laser light scattering technique (ZetaPlus, Brookhaven Instrument Corp., USA). Transmission electron microscopy (TEM) picture was taken without a heavy metal staining procedure (CM20 Microscopr TEM, Philips).

Release Experiment

Twenty mg of nanoparticles suspended in 20 ml of PBS buffer was sealed in a dialysis bag (M.W. cutoff: 10,000, Spectrapor). The dialysis bag was incubated in 30 ml of PBS buffer at 37°C. The released doxorubicin in the incubation medium was collected at pre-determined time intervals and stored frozen for quantitative analysis. The release amount was analyzed at 480 nm.

Reversed Phase High Performance Liquid Chromatography (HPLC)

The released doxorubicin and its PLGA oligomer conjugates in the medium were analyzed by a HPLC system (Waters 486) with detection at 480 nm by using the following operation conditions; PRP-3 column (4.1 mm × 150 mm, Hamilton) as a reversed phase column, a linear gradient elution of water/acetonitrile from 95/5 to 50/50; a mobile phase flow rate of 1 ml/min.

Cytotoxicity Assay

Cytotoxicities of doxorubicin and its PLGA oligomer conjugates released from nanoparticles were determined against human hepatoblastoma cell line (HepG2) obtained from Korea Research Institute of Bioscience and Biotechnology. Free doxorubicin and the released fraction from nanoparticles for 19 day incubation was used for determining the inhibition of cell growth using a tetrazolium dye (MTT) assay according to the previously established method (15). Dulbecco's modification of Eagle's MEM (DMEM) was used as a major cell growth medium and a humidified atmosphere (5% CO_2) was maintained for cell culture. HepG2 cells harvested in a logarithmic growth phase were seeded on 96 wells at a cell density of 5×10^3 cells/ml. After incubating the cells in a logarithmic phase with various concentrations of free doxorubicin and the released fraction for 72 hr, the MTT assay was performed and the percentage of cell viability was then determined.

RESULTS AND DISCUSSION

Figure 1 shows a schematic synthetic route of PLGA-doxorubicin conjugate via a carbamate linkage between a primary amine group in doxorubicin and a hydroxyl terminal end group in PLGA. The activation of the hydroxyl group in PLGA

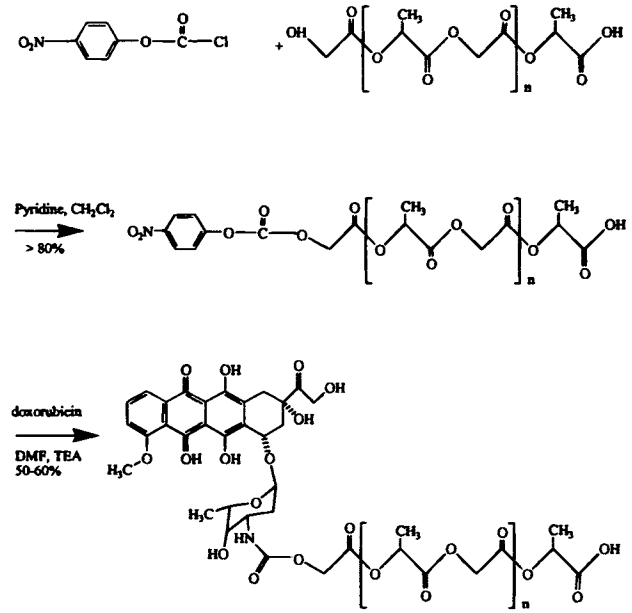

Fig. 1. Synthetic scheme of doxorubicin-PLGA conjugate.

using p-nitrophenyl chloroformate readily occurred in the presence of pyridine with a yield above 80%. The conjugation extent of doxorubicin to PLGA was 3.6% (w/w) and, in a molar basis, it was 62.1%. For the formulation of nanoparticles, it is not necessary to obtain 100% pure doxorubicin-PLGA conjugate. The PLGA-doxorubicin conjugate was analyzed by gel permeation chromatography as shown in Fig. 2. The conjugated PLGA was eluted earlier than the unconjugated PLGA, supporting that doxorubicin was conjugated to PLGA. Weight average molecular weight of the conjugate was 9210 and that of PLGA was 8020, respectively. The slight increase in molecular weight was due to the doxorubicin conjugation and the removal of low molecular weight PLGA fraction in the process of purification of doxorubicin-PLGA conjugate. From the GPC profile, it was confirmed that free doxorubicin was completely removed from the synthesized PLGA-doxorubicin conjugate.

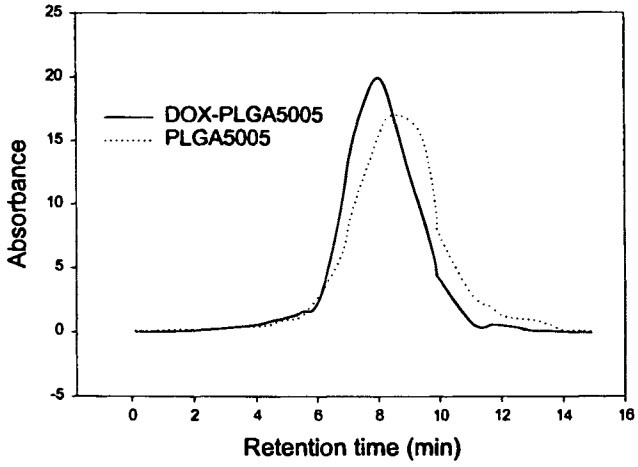

Fig. 2. Gel Permeation chromatogram of doxorubicin-PLGA conjugate. Solid line: doxorubicin conjugated polymer; dotted line: unconjugated polymer.

Figure 3 shows a transmission electron microscopy (TEM) picture of doxorubicin-PLGA conjugate encapsulated nanoparticles prepared by the spontaneous emulsion solvent diffusion method. The nanoparticles were unaggregated while maintaining an individual spherical shape. Average diameter and zeta potential of nanoparticles, as determined by a laser scattering method, was 269.7 nm and −58.1 mV for the free doxorubicin encapsulated nanoparticles, and 356.0 nm and −86.6 mV for the doxorubicin-PLGA conjugate encapsulated nanoparticles, respectively. The reduced zeta potential value for the free doxorubicin encapsulated nanoparticles was likely due to ionic interaction between a carboxylic terminal group in PLGA and a primary amino group in doxorubicin. The doxorubicin-PLGA conjugate still had negatively charged carboxylic terminal group in PLGA with concomitantly removing the positively charged primary amino group in doxorubicin. The loading percent of free doxorubicin within nanoparticles was 0.26% (w/w) with 6.7% of loading efficiency. On the other hand, the doxorubicin-PLGA conjugate encapsulated nanoparticles had 3.45% (w/w) loading percent with 96.6% efficiency. The remarkably

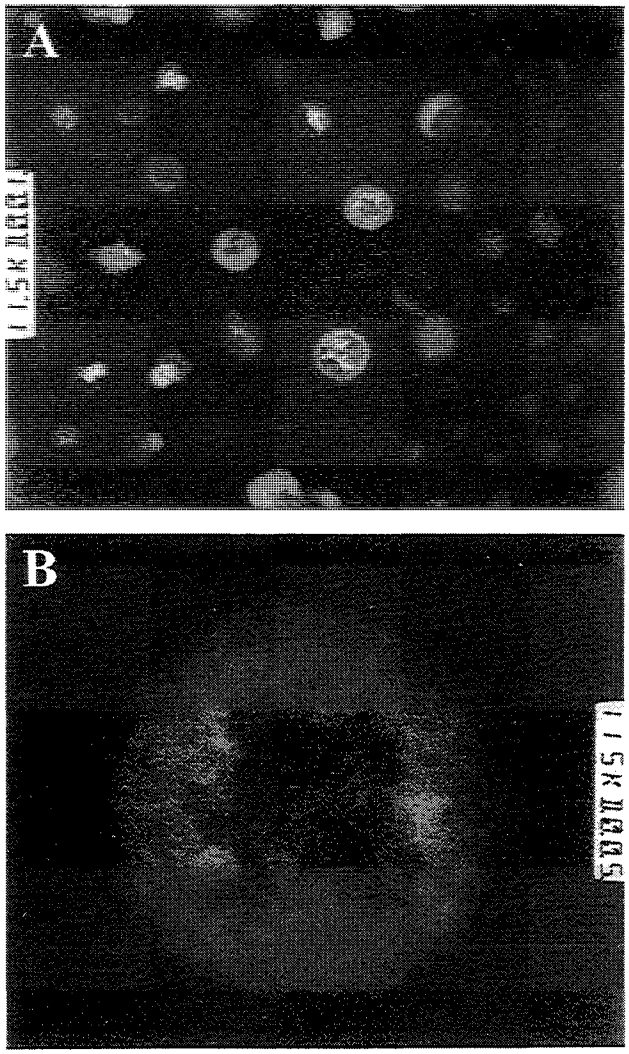

Fig. 3. Transmission electron microscopic pictures of nanoparticles containing doxorubicin-PLGA conjugate. Scale bars in A and B are 2 μm and 200 nm, respectively.

enhanced loading percent as well as its concomitantly improved efficiency for the nanoparticles containing the conjugate were clearly due to the fact that the doxorubicin-PLGA conjugate became water insoluble while free doxorubicin was moderately water soluble. It has been very difficult to encapsulate water soluble drugs within tiny nanoparticles because they freely diffuse out during the nanoparticle formation (16). In our previous report, the conjugation of hydrophilic drugs to PLGA not only dramatically increased the loading amount, but also enhanced the encapsulation efficiency for microspheres (13).

Release profiles of doxorubicin from the two nanoparticles were compared as shown in Fig. 4. The release profile of the free doxorubicn encapsulated nanoparticles exhibits a very rapid release of doxorubicin in the early incubation stage, whereas that from the conjugate encapsulated nanoparticles shows a sustained release behavior over an extended period. It should be noted that the nanoparticles containing the doxorubicin-PLGA conjugate released a mixture species of doxorubicin-PLGA oligomer conjugates because the carbamate linkage between doxorubicin and PLGA was not easily cleaved in the aqueous medium. The sustained release action was caused by the gradual chemical degradation of conjugated PLGA backbone and subsequent controlled liberation of water soluble doxorubicin-PLGA oligomer conjugates in the incubation medium. The release mechanism was not a diffusion limited process because the release pattern exhibited a near linear profile, supporting the chemically controlled release in a similar fashion to a PLGA mass erosion profile (13). Since it was found that a critical molecular weight of PLGA oligomer to be solubilized in water is around 1,000, the released doxorubicin conjugated PLGA oligomers might have up to 12–13 lactic or glycolic acid units (17). Thus, their release rate was solely dependent on how fast the conjugated PLGA chains were hydrolyzed to reach the critical MW. The released fraction from the nanoparticles collected for 19 day incubation was subjected to reversed phase HPLC analysis as shown in Fig. 5. The released fraction contains a major peak and other later eluting small peaks, which are likely to be a mixture of water soluble doxorubicin-PLGA oligomer conjugates. The hydrolytic scission of conjugated

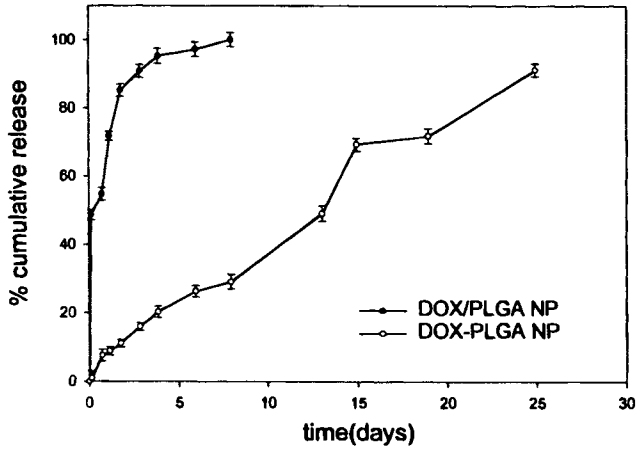

Fig. 4. Release profiles of doxorubicin from nanoparticles encapsulated with free doxorubicin (closed circle) and doxorubicin-PLGA oligomer conjugates from nanoparticles encapsulated with conjugated doxorubicin (open circle). Each data point was obtained from triplicate experiments.

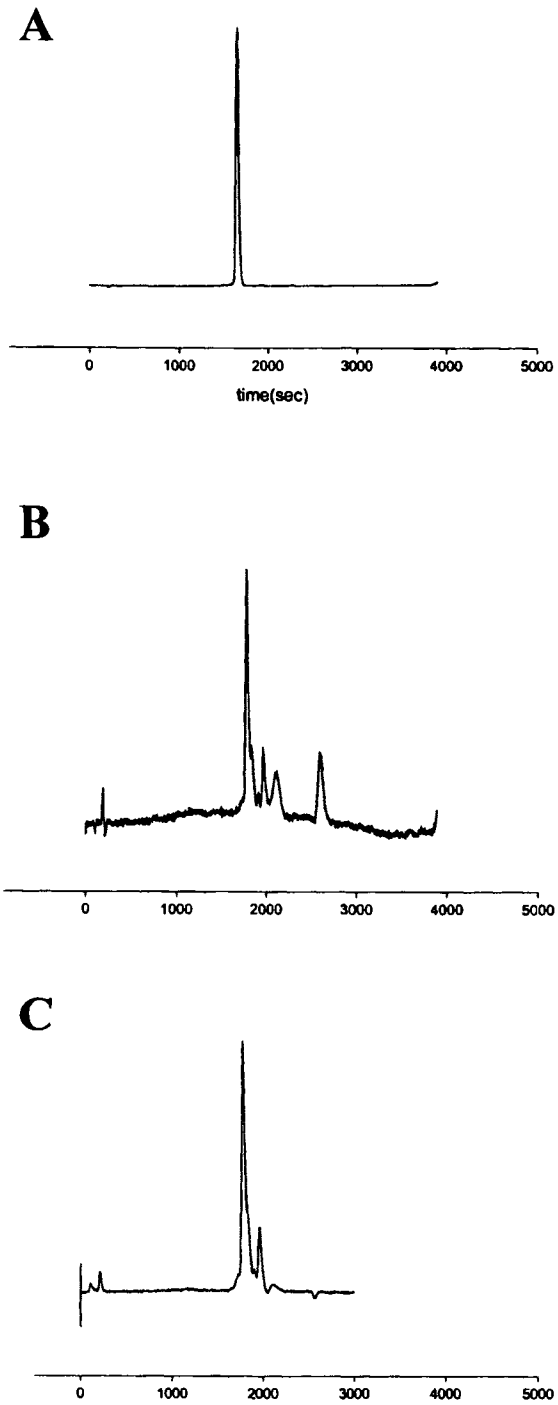

Fig. 5. Reversed phase HPLC results of the released fraction from nanoparticles. Free doxorubicin (A); released fraction for 19 days (B); further incubation of B for additional 3 days at 37°C (C).

PLGA chain unexpectedly produced fewer PLGA oligomer species as observed from the HPLC chromatogram. This can be attributed to the non-random distribution behavior of lactic and glycolic monomer units along the PLGA chain during polymerization (18). The ester linkage adjacent to the glycolic acid unit in the PLGA chain is more susceptible to hydrolysis than the lactic acid unit, resulting in the production of non-randomly cleaved PLGA oligomer species enriched with lactic

acid. Further incubation of the released fraction at 37°C for 3 additional days resulted in the growth of the major peak with concomitant disappearance of the other small peaks. This suggests the conjugated PLGA oligomers further degraded until one or two lactyl or glycolic monomer unit was still conjugated to doxorubicin via a noncleavable carbamate linkage.

The released fraction from the nanoparticles was tested to see whether a mixture of doxorubicin-PLGA oligomer conjugates still retained anti-cancer drug activity. Figure 6 shows HepG2 cell viabilities against the released fraction from nanoparticles and free doxorubicin used as a positive control. It can be seen the overall cytotoxic effect of the released fraction was slightly lower than that of free doxorubicin, presumably due to the presence of uncleaved PLGA oligomers conjugated to doxorubicin. The IC_{50} value of the released fraction was 13.7 μM and that of free doxorubicin was 8.9 μM. In the literature, the reported IC_{50} value of free doxorubicin was 7.3 μM (19). Since a mixture of doxorubicin-PLGA oligomer conjugates might have different cytotoxic activities depending on the chain length of uncleaved PLGA conjugated to doxorubicin, the above value should be regarded as an average cytotoxic activity for the released fraction during the study period.

In conclusion, this work demonstrated that by the chemical conjugation of doxorubicin to PLGA, moderately water soluble doxorubicin could be successfully encapsulated into nanoparticles with greatly enhanced doxorubicin loading and encapsulation efficiency. The nanoparticles exhibited a sustained release behavior of doxorubicin-PLGA oligomer conjugates over an extended period while maintaining comparable cytotoxicity relative to free doxorubicin. *In vivo* pharmacological activity of the released doxorubicin fraction remains to be tested in the near future. This novel formulation of doxorubicin encapsulated nanoparticles could potentially be used for passive targeting to tumor sites as well as a sustained release at the site.

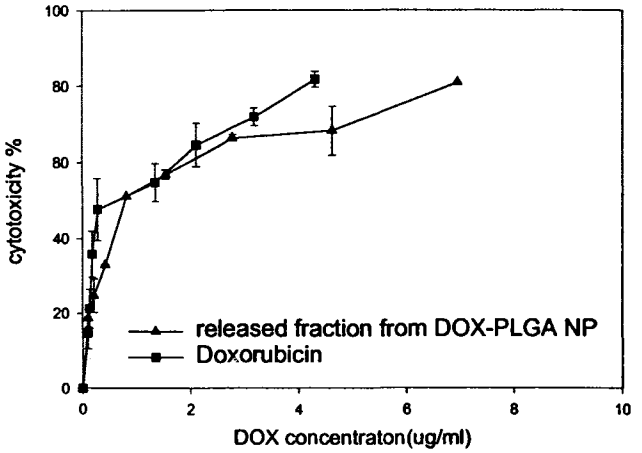

Fig. 6. Cytotoxic activity of free doxorubicin (closed square) and the released fraction from nanoparticles containing conjugated doxorubicin (closed triangle) against HepG2 cells. Each data point was obtained from triplicate experiments.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Health and Welfare (Grant #: HMP-96-D-7-1057), South Korea.

REFERENCES

1. R. Duncan. Drug-polymer conjugates: potential for improved chemotherapy. *Anti-cancer Drugs* **3**:175–210 (1992).
2. H. Maeda, M. Ueda, T. Morinaga, and T. Matsumoto. Conjugation of poly(styrene-co-maleic acid) derivatives to the antitumor protein neocarzinostatin: pronounced improvements in pharmacological properties. *J. Med. Chem.* **28**:455–461 (1985).
3. T. Minko, P. Kopeckova, V. Pozharov, and J. Kopecek. HPMA copolmer bound adriamycin overcomes MDR1 gene encoded resistance in a human ovarian carcinoma cell line. *J. Contr. Rel.* **54**:223–233 (1998).
4. H. Maeda and Y. Matsumura. Tumoritropic and lymphotropic principles of macromolecular drugs. *CRC Crit. Rev. Ther. Drug Carrier Sys.* **6**:193–210 (1989).
5. L. W. Seymour. Passive tumor targeting of soluble macromolecules and drug conjugates. *CRC Crit. Rev. Ther. Drug Carrier Sys.* **9**:132–187 (1992).
6. V. Omelyanenko, P. Kopeckova, C. Gentry, and J. Kopecek. Targetable HPMA copolymer-adriamycin conjugates. Recognition, internalization, and subcellular fate. *J. Contr. Rel.* **53**:25–37 (1998).
7. M. Yokoyama, G. S. Kwon, T. Okano, Y. Sakurai, T. Seto, and K. Kataoka. Preparation of micelle-forming polymer-drug conjugates. *Bioconjugate Chem.* **3**:295–301 (1992).
8. J. Leroux, E. Doelker, and R. Gurny. Microencapsulation: Methods and Industrial Applications, S. Benita Ed., Marcel Dekker, New York, 535–576 (1996).
9. P. Couvreur and C. Vauthier. Polyalkylcyanoacrylate nanoparticles as drug carrier: present state and perspectives. *J. Contr. Rel.* **17**:187–198 (1991).
10. Y. Morimoto, K. Sugibayashi, and Y. Kato. A antitumor effect of microsphere-entrapped adriamycin of liver metastasis of AH 7974 cells in rats. *Chem. Pharm. Bull.* **29**:1433–1439 (1981).
11. A. A. Gabizon, Y. Barenholz, and M. Bialer. Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing a polyethylene glycol-derivatized phospholipid: pharmacokinetic studies in rodents and dogs. *Pharm. Res.* **10**:703–708 (1993).
12. K. Yachi, H. Kikuchi, N. Suzuki, R. Atsumi, M. Aonuma, and Y. Kawato. Pharmaceutical and biological properties of doxorubicin encapsulated in liposomes (L-ADM): the effect of repeated administration on the systemic phagocytic activity and pharmacokinetics. *Biopharm. Drug Dispos.* **16**:653–667 (1995).
13. J. E. Oh, Y. S. Nam, K. H. Lee, and T. G. Park. Conjugation of drug to poly(D,L-lactic co-glycolic acid) for controlled release from biodegradable microspheres. *J. Contr. Rel.* **57**:269–280 (1999).
14. H. Fessi, F. Puisieux, J. P. Devissaguet, N. Ammoury, and S. Benita. Nanocapsule formation by interfacial polymer deposition following solvent displacement. *Int. J. Pharm.* **55**:R1–R4 (1989).
15. R. I. Freshney. Measurement of Viability and Cytotoxicity, Chapter 19 in *Culture of Animal Cells*, Third Edition., Wiley-Liss Inc., New York, 1994, pp. 287–307.
16. J. Kreuter. Nanoparticle-based drug delivery systems. *J. Contr. Rel.* **16**:169–171 (1991).
17. T. G. Park. Degradation of poly(D,L-lactic)microsphere: effect of molecular weight. *J. Contr. Rel.* **30**:161–173 (1994).
18. A. M. Reed and D. K. Gilding. Biodegradable polymers for use in surgery-poly(glycolci)/poly(lactic acid) homo and copolymers. 2. In vitro degradation, *Polymer* **22**:494–498 (1981).
19. E. B. Yang, W. Y. Tang, K. Zhang, L. Y. Cheng, and P. O. Mack. Norcantharidin inhibits growth of human HepG2 cell-transplanted tumor in nude mice and prolongs host survival. *Cancer Letter* **117**:93–98 (1997).