Turkish Journal of Chemistry

Volume 34 | Number 4 Article 3

1-1-2010

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Turk J Chem
34 (2010) , 509 – 516.

doi:10.3906/kim-0910-237

Preparation and characterization of anticancer drug-loaded implantable PLGA microparticles

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Received 23.10.2009

This article describes the preparation and characterization of anticancer drug-loaded poly(lactide-co-glycolide) (PLGA) microparticles. PLGA microparticles loaded with doxorubicin HCl (DOX) were prepared via o/w emulsion solvent evaporation. The release characteristics, encapsulation efficiency, size, and morphology of the PLGA microparticles were also determined. A cytotoxicity test was performed by using Glioma RG2 cancer cells to investigate the cytotoxicity of DOX-loaded PLGA microparticles. The DOX-loaded PLGA microparticles had an average diameter of 500 ± 9 nm. The DOX encapsulation efficiency and drug loading were 22.75% and 0.78%, respectively. DOX-loaded PLGA microparticles displayed a significant cytotoxicity toward the RG2 cells as compared to the unloaded PLGA microparticles.

Key Words: Doxorubicin, PLGA, RG2 cell line, microparticles

Introduction

Brain tumors constitute a profound and unsolved clinical problem, although significant strides have been made in the treatment of many other cancer types.[1] The conventional treatment for glioma has been surgical debulking of the accessible tumor from a patient's brain. Limiting tumor relapse is realized through postsurgical chemotherapy and radiotherapy over a period of time.[2] In spite of surgery, external beam radiation therapy, and systemic chemotherapy, such tumors tend to recur within centimeters of their original location.[3] The clinical application of systemic chemotherapy to brain tumors has been severely limited because potential therapeutic agents are typically unable to penetrate the blood-brain barrier (BBB),[4,5] which restricts the exchange of

solutes between the blood and the brain's extracellular fluid.[6–8] The BBB is formed by the endothelial cells of brain vessels, which, in contrast to peripheral endothelia, are joined by tight junctions.[6–8] The high systemic concentrations necessary to cross the BBB often lead to several side effects.[9]

Local drug delivery has the advantage of bypassing the BBB without increasing systemic toxicity; moreover, drugs can be delivered according to an established dose/intensity treatment to the target.[10] Therefore, the use of surgically implanted local release systems made of biodegradable polymers that deliver drugs over extended periods of time has good potential in glioma treatment.[11,12] Sustained release can be achieved using PLGA polymers and the delivery rate can be changed by varying the lactide and glycolide content.[12]

Anthracyclines belong to the most important tumor chemotherapeutics. One of the most prominent members is doxorubicin (adriamycin; adriblastin®), which is used for the treatment of solid nonhematological tumors and in part for the therapy of acute leukemias.[13] Notwithstanding the fact that DOX does not cross the BBB, several modified delivery systems have been recently developed to circumvent this obstacle and use DOX as an effective agent against brain tumors.[14–17] Although it has been shown to arrest cell growth and induce apoptosis in malignant glioma cell lines, DOX has seldom been effective in patients with brain tumors because of poor accumulation in glioma tissue following systemic administration.[18–20] To circumvent the brain's limited access of DOX, different drug delivery systems were developed,[21] including specific carrier systems such as microparticles,[22] liposomes,[23,24] nanoparticles,[6,16,25,26] chimeric peptide technology,[27] and coadministration of a drug with a P-gp modulator.[17]

Polymeric-controlled release provides a biocompatible, tunable platform in which drug loading, release rate, longevity, and form can be altered via polymer combination and processing. Furthermore, the controlled release of the drug slows elimination and increases duration of exposure. This increases the amount of drug administered without exposing the tissue to high concentrations that could cause tissue damage.[28] PLGA is used in many biomedical applications because it is an FDA-approved, biodegradable, biocompatible polymer.[29] Micro-[30–32] and nanoparticles made of PLGA were prepared for the uses of doxorubicin.[33–39]

The aim of the present study was to prepare and physicochemically characterize DOX-loaded PLGA microparticles, which could be directly injected into the brain tissue.

Experimental

Materials

Doxorubicin hydrochloride, polyvinyl alcohol (molecular weight: 30,000-70,000), acetone, and dichloromethane were purchased from Sigma Chemical (Missouri, USA). PLGA (Resomer RG502) was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany). Rat glioma cell line (RG2 cell line) was obtained from the American Type Culture Collection (Virginia, USA). Fetal bovine serum and penicillin G sodium-streptomycin sulfate solution were purchased from Biochrom (Berlin, Germany). Dulbecco's Modified Eagle's Medium, L-glutamine, and sodium pyruvate were provided by Biological Industries Ltd. (Kibbutz Beit Haemek, Israel).

Preparation of microspheres

PLGA microparticles were prepared by using a modified version of an o/w single-emulsion solvent evaporation process.[40] In brief, the organic phase consisted of PLGA polymer in an acetone-dichloromethane mixture (0.5:1). The aqueous phase contained PVA solution and drug, dissolved. The organic phase was emulsified with the aqueous phase by using an Ultra-Turrax model T25 (IKA Labortechnik, Germany) at 13,500 rpm in an ice bath for 3 min. Then the organic mixture was removed rapidly by evaporation under reduced pressure at 37 °C. The particles were centrifuged at 13,500 rpm for 40 min, washed 3 times in distilled water, and freeze-dried.

Surface morphology and particle size

The surface morphology of the PLGA microparticles was examined by scanning electron microscopy (SEM, JEOL-SEM 1200 EX) (JEOL Ltd., Tokyo, Japan). Microspheres were mounted on metal stubs with conductive silver paint and then sputtered with a layer of gold at 150 Å thickness using a Bio-Rad apparatus. Particle size distribution of the PLGA microparticles was determined by laser diffractometry using a Malvern Zetasizer 3000HS (Malvern Instruments Ltd., Worcestershire, United Kingdom).

Determination of drug loading

The drug-loaded microparticles were separated from the aqueous suspension medium by ultracentrifugation at 13,500 rpm and 4 °C for 40 min. The amount of free drug was measured in the clear supernatant using HPLC-DAD. Reversed phase chromatography for the HPLC method was conducted by using a Phenomenex Bondclone C_{18} column with an isocratic mobile phase consisting of acetonitrile and water (1:1). The effluent was monitored on a DAD detector at 490 nm. A linear response ($r > 0.99$) was observed over the range of 25-200 μg mL^{-1}

In vitro release study

The in vitro drug release studies of the DOX-loaded PLGA microspheres were carried out in phosphate-buffered saline (PBS, pH 7.4). The freeze-dried microspheres (1 mg) were dispersed in 1 mL of PBS and placed in a shaker bath at 50 rpm at 37 ± 0.5 °C. The in vitro release of DOX was quantified by using HPLC-DAD under conditions similar to those described above.

Cell culture

RG2 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 0.584 g L^{-1} L-glutamine, 0.11 g L^{-1} sodium pyruvate, 50 U mL^{-1} penicillin G sodium, and 50 μg mL^{-1} streptomycin sulfate at 37 °C in a humidified incubator containing 5% CO_2. Cells were grown in 75 cm^2 culture flasks. Confluent cell monolayers were trypsinized cells plated into 96 well, flat-bottomed plates at 5 × 10^3 cells/100 μL in each well, incubated overnight at 37 °C in a CO_2 incubator. The following day, cells were treated with DOX solutions, unloaded PLGA microspheres, and DOX- loaded PLGA microspheres in 20 μL of complete medium and incubated for 48 h in the incubator. Next, 25 μL of MTT solution (1 mg mL^{-1})

was added to each well and left for 4 h. The medium was then removed from each well and 200 μL of DMSO was added to each well. The plate was placed on a shaker for 2 min to dissolve the formazan in the DMSO. Following overnight incubation at 37 °C, the optical densities were measured at 570 nm using a microplate reader (Spectramax Plus, Molecular Devices, Berkshire, United Kingdom).

Results and Discussion

Doxorubicin has not yet been used successfully to treat malignant gliomas, while the in vitro potency of doxorubicin is remarkable and its current indications in treating peripheral tumors have proven efficacious.[41] Systemically administered doxorubicin has shown poor penetration of the BBB and attempts to improve drug delivery via cerebrospinal infusion have not been very successful. Underlying the failure of these attempts has been the fundamental limitation in achieving therapeutic concentrations of doxorubicin in the CNS while minimizing systemic adverse side effects.[41] The failure of conventional systemic drug delivery for glioma has motivated more direct approaches to drug delivery. Direct intracranial drug delivery would eliminate the need for a chemotherapeutic agent to cross the BBB. Local delivery to brain tumors has already provided a modest increase in survival when it is used in addition to surgery and radiotherapy.[28]

Polymer nanospheres, microspheres, and wafers of varying sizes are made via emulsion solvent evaporation, salt leaching, and other methods. Implantable PLGA matrices that are loaded with chemotherapeutics are currently under development.[28] In this study, the emulsion solvent evaporation method was successful in producing DOX-loaded PLGA microparticles for local application to the brain, employing PVA as a stabilizer. SEM images revealed that the PLGA microparticles had spherical and smooth surfaces (Figure 1).

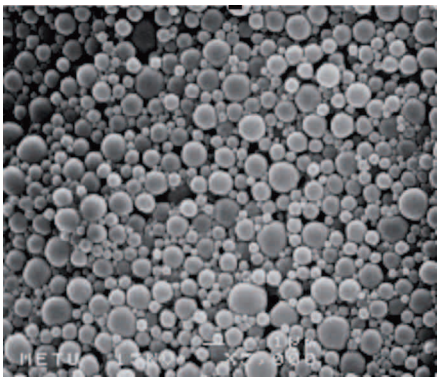

Figure 1. Scanning electron microscopic photograph of the PLGA microparticles.

The DOX-loaded PLGA microparticles had an average diameter of 500 ± 9 nm. The DOX encapsulation efficiency was 22.75%, which leads to a final DOX loading value of 0.78%. The key parameter for the successful entrapment of the drug is its insolubility in the organic polymer solution. However, as DOX is insoluble in organic solvents, the unencapsulated drug might be lost when an organic solvent evaporates, and consequently the drug is lost into the aqueous phase.[31,42]

The encapsulation of DOX into PLGA has been reported in previous studies. For example, Lin et al.[31] described the in vitro release of DOX from PLGA-based microparticles fabricated using the spray-drying

technique. They reported a 60%-70% total release on day 1 from their single-wall microparticles. In addition, Tan et al.[43] prepared double-walled microspheres containing DOX to investigate drug release and reported an initial release of 0.89%-12.89% within the first 6 h. Furthermore, Lesniak et al.[41] devised a strategy of delivering DOX locally and in a controlled manner to achieve a therapeutic concentration of DOX. Separately, DeFail et al.[30] investigated the controlled release of bioactive DOX from PLGA microspheres embedded within gelatin scaffolds. In their study, at day 1, the microspheres alone released 10.8% ± 6.0% of the total DOX released, and the DOX released from the gelatin constructs was 7.6% ± 0.5% at day 1. The release of DOX was then significantly delayed during days 5-16, when the PLGA microspheres were incorporated into the gelatin.

In other studies, Weinberg et al.[44] evaluated the antitumor efficacy and local drug distribution from DOX-containing PLGA implants for intratumoral treatment of liver cancer in a rabbit model. In vitro DOX-containing implants were found to release a total of 2.07 ± 0.05 mg of drug over the 8-day period, corresponding to (71.3 ± 1.7)% of the total drug loading. The release half-time was approximately 4 h, and the vast majority of the doxorubicin, 1.98 ± 0.03 mg, was released in the first 24 h. On the other hand, Manome et al.[45] prepared implantable, biocompatible, and biodegradable devices bearing an anticancer drug for local therapy for patients with malignant disorders. With the aim of treating brain tumors, especially gliomas, a membranous sheet containing DOX was produced by copolymerization to PLGA. When release of the drug from the sheet was measured, sustained release continued until day 34. The data contrasted with the burst release from material containing a higher proportion of the drug.

In the present study, the DOX release profile was obtained in PBS, pH 7.4, and the release study was carried out in sink conditions. The cumulative in vitro release of DOX from the PLGA microparticles is shown in Figure 2. In general, the faster release from the smaller microparticles seems to be due to the larger surface area and shorter length of the diffusion path.[46] The common feature in these profiles was the initial fast release (day 1) of DOX, which was poorly entrapped in the polymer matrix, and about 60% of the DOX was released after the first day.

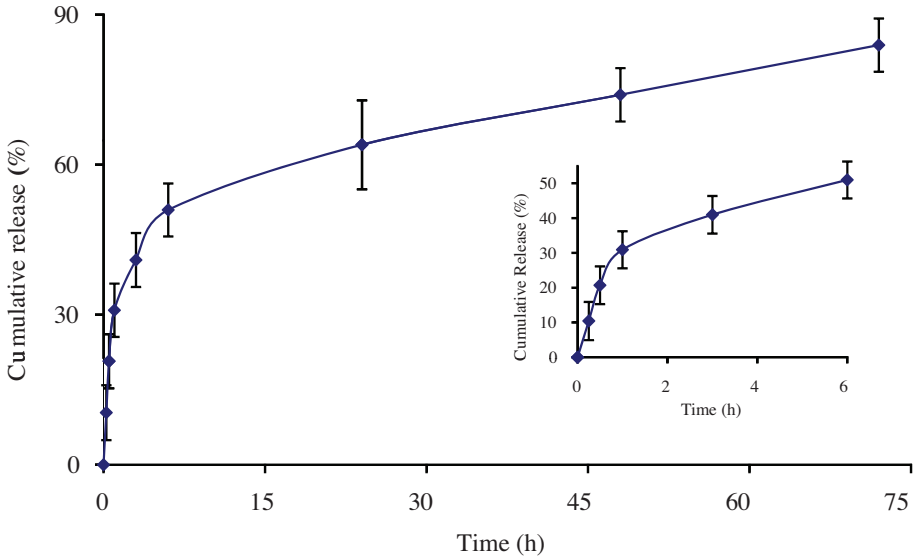

Figure 2. Release profiles of DOX-loaded PLGA microparticles (mean ± SD, n = 3).

Malignant tumor cells were incubated with free DOX and DOX-loaded and unloaded PLGA microparticles. The cytotoxicity of free doxorubicin and doxorubicin-loaded PLGA microparticles was analyzed by using the MTT assay. In this assay, the MTT salt is added to the media and reduced by mitochondrial dehydrogenases to the water-insoluble MTT-formazan upon internalizing into the cells. The absorbance is recorded and, hence, the extent of the viability is defined as the relative reduction of the absorbance, which in turn is directly correlated with the amount of viable cells in relation to the cell control. In Figure 3, the MTT data shows a concentration-dependent decrease in cell line. De Juan et al.[21] reported that the decrease in viability was most pronounced at DOX concentrations below 1 μg mL^{-1}. DOX-loaded PLGA microparticles (equivalent to 7 μg of DOX) displayed a significant cytotoxicity toward the RG2 cells as compared to unloaded PLGA microparticles (Figure 3). The viability of RG2 cells did not change significantly up to the highest concentration of 7 μg mL^{-1}. A similar conclusion was reported in a previous paper.[21]

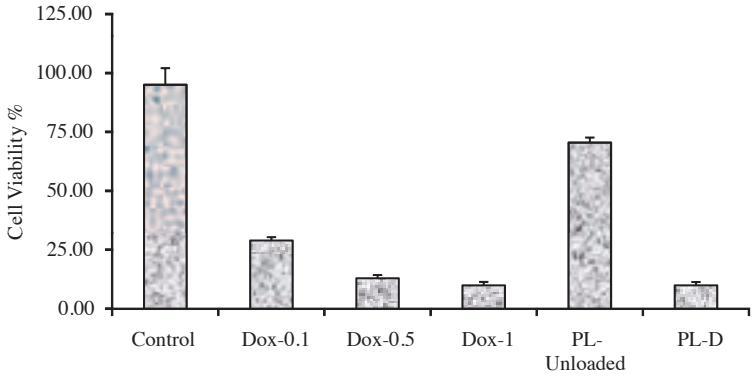

Figure 3. Viability of RG2 according to the MTT assay after 48 h incubation with different concentrations of several DOX standards (0.1, 0.5, and 1 μg mL^{-1}), DOX-loaded PLGA microparticles (PL-D), and unloaded PLGA microparticle formulations (PL-unloaded) (mean $\pm$ SD, n = 3). Groups were compared with the one-way ANOVA/Tukey test. Statistical significance was set at $P < 0.05$. $P < 0.05$ between the control and other groups; the only groups that do *not* differ from each other at $\alpha = 0.05$ are groups DOX-1 and PL-D ($P > 0.05$).

Conclusion

DOX-loaded PLGA-microparticles were prepared using an o/w single-emulsion solvent evaporation method and characterized in vitro. Importantly, these devices can be directly injected into the brain tissue, overcoming the restriction that doxorubicin cannot cross the BBB after systemic administration. Thus, this type of controlled drug delivery system might be helpful for improving the local treatment of brain tumors. This work can be considered for further studies on evaluation of the in vivo efficacy and the brain's regional distribution of DOX-loaded PLGA microparticles using mature male Sprague-Dawley rats.

Acknowledgement

This study was supported by Ataturk University Research Foundation (project number: 2007/56).

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