Biodegradable polymeric micelles composed of doxorubicin conjugated PLGA–PEG block copolymer

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Abstract

Biodegradable polymeric micelles containing doxorubicin in the core region were prepared from a di-block copolymer composed of doxorubicin-conjugated poly(ε-lactic-co-glycolic acid) (PLGA) and polyethyleneglycol (PEG). The di-block copolymer of PLGA–PEG was first synthesized and the primary amino group of doxorubicin was then conjugated to the terminal hydroxyl group of PLGA, which had been pre-activated using p-nitrophenyl chloroformate. The resulting polymeric micelles in aqueous solution were characterized by measurement of size, drug loading, and critical micelle concentration. The micelles containing chemically-conjugated doxorubicin exhibited a more sustained release profile than PEG–PLGA micelles containing physically-entrapped doxorubicin. The cytotoxic activity of the micelles against HepG2 cells was greater than free doxorubicin, suggesting that the micelles containing conjugated doxorubicin were more effectively taken up cellulary, by an endocytosis mechanism rather than by passive diffusion. Confocal microscopic observation and flow cytometry analysis supported the enhanced cellular uptake of the micelles. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Doxorubicin; Biodegradable; Micelles; PLGA; Endocytosis

1. Introduction

Amphiphatic block copolymers self-assemble into polymeric micelles in aqueous solution, and potentially can be used as parenteral drug delivery systems [1–5]. Various polymeric micelles have been used for the solubilization of water insoluble drugs within the interior region of micelles. The size of polymeric micelles, approximately 50 nm, not only makes them ideal drug delivery carriers for escaping from renal exclusion and the reticulo-endothelial system, but also enhances their vascular permeability [2]. A few studies of polymeric micelles containing anti-cancer drugs have proposed the possibility of passively targeting solid tumors, compared to the extensive research on drug–polymer conjugates and liposomes [6,7]. For instance, di-block copolymers composed of poly(aspartic acid)–poly(ethyleneglycol) have been used to physically entrap or chemically conjugate doxorubicin within the polyanionic core of micelles, and have resulted in an effective suppression of tumor growth when injected in vivo. Intravenously-injected micelles diffused through loose-
ly packed endothelial cell junctions of blood capillaries in the vicinity of tumor tissue and then accumulated in the solid tumor by `enhanced permeation and retention’ (EPR) effect [2]. Moreover, enhanced uptake of polymeric drugs within tumor cells was believed to occur by increased endocytotic transport and by circumventing the multi drug resistance (MDR) effect [7,8].

Most polymeric micelles are composed of hydrophobic segments as the internal core and hydrophilic segments as a surrounding corona [9]. The hydrophobic core serves as a reservoir for hydrophobic drugs, whereas the hydrophilic shell affects their pharmacokinetic behavior. In particular, biodegradable polymeric micelles have received much attention. Various aliphatic polyesters such as poly(t-lactic acid) (PLA), poly(\(\text{DL-}\)lactic-co-glycolic acid) (PLGA), and poly(\(\varepsilon\)-caprolactone) (PCL) have been combined with a hydrophilic PEG segment to produce an A–B type di-block copolymer structure. For example, di-block copolymers of PLA–PEG and PCL–PEG have been synthesized by ring opening polymerization of \(\text{L-}\)lactide and \(\varepsilon\)-caprolactone, respectively, in the presence of methoxy-terminated PEG [10,11]. These polymeric micelles, however, have a relatively low loading of moderately watersoluble drugs due to a limited partitioning of more hydrophilic drugs into the hydrophobic polymer phase. This can be regarded as one of the major limitations of polymeric micelle systems.

In this paper, we describe a doxorubicin delivery system using a di-block copolymer composed of doxorubicin conjugated PLGA and PEG. It was expected that a greater amount of doxorubicin could be loaded into the inner PLGA core of these polymeric micelles, relative to the physical entrapment of free doxorubicin. A mixture of \(\text{DL-}\)lactide and glycolide, molar ratio of 50/50, was ring opening polymerized in the presence of monomethoxy PEG 2000. The terminal hydroxyl group of the PLGA chain end was utilized to conjugate doxorubicin after activation with \(p\)-nitrophenyl chloroformate. The polymeric micelles were characterized and their cytotoxicity against HepG2 cells was investigated. Confocal microscopy and flow cytometry studies were also carried out to elucidate the transport of these micelles within cells.

2. Experimental methods

2.1. Materials

\(\text{DL-}\)lactide and glycolide, purchased from Purac (Gorinchem, The Netherlands), were re-crystallized from ethyl acetate before use. Monomethoxy poly-(ethylene glycol) (mPEG), \(M_w\) 2000, stannous octoate, doxorubicin, \(p\)-nitrophenyl chloroformate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). Human hepatoblastoma cell line (HepG2) was obtained from Korean Cell Line Bank (KCLB). All other chemicals were of analytical grade.

2.2. Preparation of doxorubicin conjugated PLGA–PEG block copolymer

\(\text{DL-}\)lactide (14.4 g), glycolide (11.6 g), and mPEG (8 g) in a bottle-neck flask were heated to 140°C under nitrogen atmosphere for complete melting. The molar ratio of \(\text{DL-}\)lactide and glycolide was 1:1. Then, 0.05% (w/w) stannous octoate was added, and the temperature of the reaction mixture was raised to 180°C and maintained for 3 h. The polymerization was carried out under vacuum. The polymer was recovered by dissolution in methylene chloride followed by precipitation in ice-cold diethyl ether [10]. The precipitate was filtered and dried under reduced pressure. To PLGA–PEG di-block copolymer (3 g) dissolved in methylene chloride (30 ml), dropwise \(p\)-nitrophenyl chloroformate (80 mg) and pyridine (63 mg) were added; (PLGA–PEG–\(p\)-nitrophenyl chloroformate–pyridine, stoichiometric molar ratio: 1/2/4) at 0°C. After 3 h at room temperature under nitrogen, the activated PLGA–PEG di-block copolymer was recovered by precipitation in ice-cold diethyl ether. The precipitate was filtered and dried under reduced pressure. The activated PLGA–PEG (0.25 g) in dimethylformamide (7.5 ml) was reacted with doxorubicin (10 mg) in the presence of triethylamine (6.75 mg) for 24 h at room temperature under nitrogen (stoichiometric molar ratio of activated PLGA–PEG–doxorubicin–triethylamine: 1/1/4). The doxorubicin–PLGA-b–PEG (DOX–PLGA–PEG) was purified as polymeric micelles by adding...
the organic phase to deionized water, followed by
dialyzing three times against deionized water for 4 h
(Spectra/Por 7, MWCO 10 000). After the dialysis,
DOX–PLGA–PEG was freeze dried and stored at
−20°C. The doxorubicin content was determined to
be 36.5 mol% by measuring the UV absorbance of a
DMSO solution at 480 nm. A calibration curve was
constructed using different concentrations of free
doxorubicin in DMSO.

2.3. Preparation of doxorubicin loaded micelles

DOX–PLGA–PEG (100 mg) in acetone (10 ml)
was added to PBS buffer (100 ml, pH 7.4). Spontan-
eous formation of micelles was confirmed by the
observation of a transparent reddish solution. For the
preparation of PLGA–PEG micelles containing
physically entrapped doxorubicin, PLGA–PEG (97.8
mg) and doxorubicin (2.2 mg) co-dissolved in
acetone (10 ml) were added to PBS buffer (100 ml).
The resulting micelles contained a large fraction of
unentrapped doxorubicin, detected by reversed phase
HPLC. The doxorubicin-loaded micelles were exten-
sively dialyzed to remove unentrapped doxorubicin
before further use.

2.4. Size distribution and doxorubicin loading
amount and efficiency

To confirm the formation of micelles, size dis-
tribution was measured using a laser light scattering
technique (ZetaPlus, Brookhaven Instrument Corp.,
USA). The loading amount and loading efficiency
were calculated based on the weight ratio of the
loaded amount of doxorubicin to the amount of
micelles, and the percent ratio of the amount of
doxorubicin incorporated into the micelles to an
initial amount used, respectively [12]. The doxorubi-
cin-loaded micelles were freeze-dried, dissolved in
DMSO, and then used for the determination of
doxorubicin in the micelles as described above.

2.5. Determination of critical micelle concentration
(CMC) of DOX–PLGA–PEG

The CMCs of the doxorubicin conjugated di-block
copolymer and unconjugated di-block copolymer
were determined using pyrene as an extrinsic probe
[13]. Briefly, pyrene (4 μg) pre-dissolved in methyl-
eine chloride (10 ml), was added to a test tube and
the solvent was evaporated. Deionized water (5 ml)
was added to the test tube and different amounts of
doxorubicin-conjugated and unconjugated di-block
copolymers dissolved in acetone was added to the
aqueous phase. The solution was incubated at room
temperature with mild stirring for complete evapora-
tion of the organic solvent to induce the formation
of micelles. The fluorescence intensity of the solution
was measured at room temperature using a spec-
trofluorometer (Shimadzu RF5301PC, Japan). The
concentration of pyrene was fixed at 0.2 μM and the
final concentration of DOX–PLGA–PEG was varied
from 10 to 1000 μM. Emission spectra were ob-
tained at 377, 383 and 392 nm, which were de-
determined from the scanning emission spectrum of
each sample from 300 to 600 nm, fixing the excita-
tion wavelength at 339 nm. The CMC was de-
determined by taking a mid-point of the copolymer
concentration at which the relative emission fluores-
cence intensity ratio measured at 383 nm to 377 nm
was varied. Since the emission wavelength of doxo-
ubicin appearing around 530 nm was far from those
of pyrene, the CMC values could be determined
without interference from the presence of the conju-
gated doxorubicin within the micelles.

2.6. Drug release experiment

Twenty mg of micelles in PBS buffer (20 ml)
were sealed in a dialysis bag (M_w cutoff: 10 000,
Spectra/por), and incubated in PBS buffer (30 ml) at
37°C. The doxorubicin released into the incubation
medium was collected at pre-determined time inter-
vals and stored frozen for quantitative analysis. The
release amount was determined from the absorbance
at 480 nm.

2.7. Determination of DOX–PLGA micelles
transported into HepG2 cells

HepG2 cells were used as a model cancer cell line
for the examination of endocytosis. HepG2 cells,
5×10^3 cells/ml, were seeded onto a 100 mm culture
dish or cover glass pre-soaked in culture medium and after 24 h DOX–PLGA–PEG micelles or free doxorubicin were added to the culture medium, both of which had the same doxorubicin concentration of 170 \(\mu\)M. The loading amount of doxorubicin in DOX–PLGA–PEG micelles was taken into account to adjust the doxorubicin concentration. After 6 h, the cells on the cover glass were thoroughly washed three times with PBS and examined by a confocal microscopy (Carl Zeiss LSM5100, Germany) at an excitation wavelength of 488 nm. An emitting signal was detected at 515 nm. In order to quantify the amount of doxorubicin within the cell, the cells were harvested and analyzed by flow cytometry (Becton Dickinson FACSCalibur, USA), and the gate was arbitrary set for the detection of green fluorescence (150<FL1-H<400, 0<FSC<200, linear scale). The relative fluorescence intensity of HepG2 cells incubated with DOX–PLGA–PEG micelles was calculated using those incubated with free doxorubicin as a control, 100%. The reason for using such a high concentration of doxorubicin in this experiment, although it was high enough for inducing cell death, was to clearly visualize the confocal microscopic image and to readily observe the FACS results. The exposure time, 6 h, was sufficient to observe the endocytic behavior of the micelles within HepG2 cells, but it was too short to kill the HepG2 cells. Cell death was observed by a trypan blue exclusion method.

### 3. Results and discussion

The synthesis of DOX–PLGA–PEG is shown in Fig. 1. A di-block copolymer of PLGA–PEG was first prepared by ring opening polymerization of \(DL\)-lactide and glycolide in the presence of mPEG (\(M_w\) 2000) [10]. The \(M_n\) of the copolymer and the molar ratio of \(DL\)-lactide to glycolide were determined by \(^1\)H NMR to be 9600 and 50:50, respectively. The \(M_n\), \(M_w\) and polydispersity were determined by GPC to be 13 000, 23 000 and 1.7, respectively. GPC showed a single peak that was eluted earlier than those of PLGA and PEG, confirming the successful synthesis of the PLGA–PEG di-block copolymer and the absence of PLGA and PEG homopolymers. The \(T_g\) was determined by DSC to be 1.2°C.

Doxorubicin was conjugated to PLGA via a carbamate linkage between the primary amine group in doxorubicin and the terminal hydroxyl group in PLGA, activating the latter with \(p\)-nitrophenyl chloroformate. The conjugation of doxorubicin to PLGA–PEG was confirmed by the GPC elution
profiles of the PLGA–PEG and DOX–PLGA–PEG, which were monitored by refractive index and fluorescence detectors, respectively. A single peak was eluted at the same retention time using both refractive index and fluorescence detectors, without free PEG and doxorubicin peaks. This indicated that DOX–PLGA–PEG was successfully purified by dialysis after the conjugation. However, it should be mentioned that the sample consisted of 36.5 mol% DOX–PLGA–PEG and 63.5 mol% PLGA–PEG.

The CMC of DOX–PLGA–PEG was determined using pyrene as an extrinsic probe according to the previously published method [13]. As shown in Fig. 2, the CMC of DOX–PLGA–PEG micelles was approximately 0.1 μg/ml, similar to the CMC of doxorubicin unconjugated PLGA–PEG micelles. Thus, the conjugation of doxorubicin to PLGA–PEG did not affect the micelle-forming ability of PLGA–PEG. This can be attributed to the relatively small molecular size of doxorubicin compared to the size of the hydrophobic PLGA chain segment in the block copolymer. This result also suggests that moderately hydrophobic doxorubicin, conjugated to the terminal end of PLGA chain, may be located in the hydrophobic core of the micelles without disturbing the micellar structure.

The physical characteristics of DOX–PLGA–PEG micelles, specifically the effective diameter, and the doxorubicin loading amount and efficiency, are compared in Table 1 with those of PLGA–PEG micelles that were physically loaded with free doxorubicin. The sizes of DOX–PLGA–PEG and PLGA–PEG micelles, as determined by a dynamic laser scattering method, were both about 60 nm in diameter. This finding is consistent with the CMC result that doxorubicin conjugation to PLGA did not effect the micelle-forming capability. The slightly increased diameter (61.5 nm) of DOX–PLGA–PEG, relative to the diameter (58.2 nm) of PLGA–PEG, may be attributed to doxorubicin conjugation. The loading amount and efficiency of free doxorubicin within polymeric PLGA–PEG micelles were 0.51% (w/w) and 23.2%, respectively. On the other hand, those of the DOX–PLGA–PEG micelles were 2.18% (w/w) and 99.1%, respectively. The enhanced loading amount and efficiency for the micelles prepared from DOX–PLGA–PEG were clearly attributable to the fact that doxorubicin conjugated to PLGA–PEG was forcefully located within the core of hydrophobic PLGA chains. In the case of physical entrapment, the moderately water-soluble doxorubicin can escape into the aqueous phase during the self-assembling process. The partition behavior of doxorubicin between the water phase and the PLGA bulk phase will determine the loading amount and efficiency. The limitation of drug loading efficiency within polymeric micelles can be overcome by the conjugation approach.

The release of doxorubicin from DOX–PLGA–PEG was

<table>
<thead>
<tr>
<th>Doxorubicin loading</th>
<th>Effective diameter (nm)</th>
<th>Theoretical loading (w/w, %)</th>
<th>Actual loading (w/w, %)</th>
<th>Loading efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physically entrapped (PLGA–PEG)</td>
<td>58.21±6.21</td>
<td>2.20</td>
<td>0.51±0.07</td>
<td>23.18±3.18</td>
</tr>
<tr>
<td>Conjugated (DOX–PLGA–PEG)</td>
<td>61.48±7.17</td>
<td>2.20</td>
<td>2.18±0.04</td>
<td>99.09±1.81</td>
</tr>
</tbody>
</table>
PEG micelles and PLGA–PEG micelles containing physically entrapped doxorubicin is shown in Fig. 3. While the DOX–PLGA–PEG micelles showed sustained release of up to 50% of the drug load over 2 weeks, release from PLGA–PEG micelles containing non-conjugated doxorubicin lasted only for 3 days. The sustained release of DOX–PLGA–PEG micelles is attributed to the gradual hydrolysis of the conjugated PLGA backbone and subsequent controlled liberation of water-soluble DOX–PLGA oligomer conjugates in the incubation medium [14]. The sustained release of doxorubicin from the DOX–PLGA–PEG micelles was similarly observed in formulations of PLGA microspheres and nanoparticles that were prepared by the conjugation of various hydrophilic drugs to PLGA [12,15,16]. Since the carbamate linkage between doxorubicin and PLGA chain is hydrolyzed very slowly, the released doxorubicin fraction contains a mixture of doxorubicin–PLGA oligomer species. Consequently, the doxorubicin released from the micelles may have slightly modified properties. This concern was discussed in the previous reports [15]. However, it was found that the released doxorubicin fraction from doxorubicin-conjugated PLGA nanoparticles exhibited a comparable in vitro cytotoxic effect to free doxorubicin against HepG2 cells [15]. Thus, it was of interest to study whether the DOX–PLGA–PEG micelles had a comparable cytotoxic effect.

The cytotoxicities of free doxorubicin and DOX–PLGA–PEG micelles against HepG2 cells are compared as shown in Fig. 4. In this experiment, freshly prepared DOX–PLGA–PEG micelles were directly used to test the in vitro cytotoxicity. For comparison, the doxorubicin concentration in DOX–PLGA–PEG was adjusted to be the same as that of free doxorubicin. It can be seen that DOX–PLGA–PEG micelles are about 10-fold more cytotoxic than free doxorubicin. This remarkable result can be explained by the enhanced uptake of doxorubicin conjugated PLGA–PEG micelles relative to free doxorubicin. The uptake of DOX–PLGA–PEG micelles by HepG2 cells was investigated qualitatively and quantitatively using a confocal microscopy and a flow cytometry, respectively. As shown in Fig. 5, HepG2 cells incubated with DOX–PLGA–PEG micelles emitted more intense fluorescence than those incubated with free doxorubicin. The enhanced uptake of DOX–PLGA–PEG micelles can be attributed to their facilitated endocytotic transport, relative to passive diffusion of free doxorubicin through the cell membrane. It is likely that the increased uptake of micelles occurred by a ‘fluid-phase endocytotic mechanism’ because there is no specific affinity between the outer PEG chains of the micelles and negatively charged plasma membrane of the cells. Additionally, the multi-drug resistance (MDR) effect might play a role in reducing the accumulation of free doxorubicin within cells: free doxorubicin molecules transported within cells are likely to be pumped
back out of the cytosol by the \( p \)-glycoprotein pump expressed in the membrane of cancerous cells. From this standpoint, cellular delivery of doxorubicin based on polymeric micelles is an attractive way to circumvent the MDR effect. In the previous study of PLGA nanoparticles formulated with PLGA conjugated doxorubicin, a similar enhanced uptake of doxorubicin by HepG2 cells was observed [12,16].

While HepG2 cells incubated with free doxorubicin emitted fluorescence mainly at the nucleus, those with DOX–PLGA–PEG micelles showed some fluorescence in the cytosol as well as intense fluorescence at the nucleus (Fig. 5).

Flow cytometry also confirmed the enhanced uptake of DOX–PLGA–PEG micelles. As shown in Fig. 6, HepG2 cells incubated with DOX–PLGA–PEG micelles showed a similar enhanced uptake of doxorubicin by HepG2 cells was observed [12,16].
PEG micelles emitted higher fluorescent intensity than those with free doxorubicin. When the fluorescence intensities of the two samples within the arbitrary set gated region (150<FL1-H<400, 0<FSC<200) were compared, HepG2 cells incubated with DOX–PLGA–PEG micelles showed five times more fluorescence intensity than those with free doxorubicin in the selected region. Thus, it can be said that DOX–PLGA–PEG micelles are taken up by HepG2 cells to a much greater extent than free doxorubicin.

4. Conclusions

A higher loading of doxorubicin in polymeric micelles could be obtained by conjugation of doxorubicin to a di-block copolymer of PLGA–PEG. There was no change in critical micelle concentrations of PLGA–PEG with and without conjugation to doxorubicin. While DOX–PLGA–PEG micelles showed a sustained release profile over 2 weeks, PLGA–PEG micelles containing physically entrapped doxorubicin exhibited a burst release at the initial stage of incubation. DOX–PLGA–PEG micelles were more cytotoxic against HepG2 cells than free doxorubicin. This was ascribed to the greater uptake of DOX–PLGA–PEG micelles by HepG2 cells, compared to free doxorubicin, resulting from enhanced endocytosis of DOX–PLGA–PEG micelles.

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References