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RESEARCH PAPER

Accelerated Blood Clearance of PEGylated PLGA Nanoparticles Following Repeated Injections: Effects of Polymer Dose, PEG Coating, and Encapsulated Anticancer Drug

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ABSTRACT

Purpose To investigate accelerated blood clearance (ABC) induction upon repeated injections of PLGA-PEG nanoparticles as a commonly used polymeric drug carrier.

Methods Etoposide-loaded PLGA-PEG NPs were developed and administered as the test dose to rats pre-injected with various NP treatments at certain time intervals. Pharmacokinetic parameters of etoposide and production of anti-PEG IgM antibody were evaluated.

Results A notable ABC effect was induced by a wide range of polymer doses (0.1 to 20 mg) of empty NPs, accompanied by IgM secretion. However, a further increase in polymer dose resulted not only in the abrogation of the observed ABC induction but also in distinctly a higher value for AUC of the NPs relative to the control. The data from the PEG-negative group verified the fundamental role of PEG for ABC induction. The first injection of etoposide-containing PEGylated nanoparticles (a cell cycle phase-specific drug) produced a strong ABC phenomenon. Three sequential administrations of etoposide-loaded NPs abolished ABC, although a high level of IgM was still detected, which suggests saturation with insignificant poisoning of immune cells.

Conclusion The presented results demonstrate the importance of clinical evaluations for PLGA-PEG nanocarriers that consider the administration schedule in multiple drug delivery, particularly in cancer chemotherapy.

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KEY WORDS accelerated blood clearance (ABC) · anti-PEG IgM antibody · etoposide · pharmacokinetics · PLGA-PEG nanoparticles

INTRODUCTION

Polymeric nanoparticles (NPs) have been extensively investigated as carrier systems for targeted drug delivery to tumors. They offer a promising means of modifying the original pharmacokinetics and biodistribution of the encapsulated chemotherapeutic drug, raising its concentration in the target tissue and limiting its interaction with healthy cells, consequently improving the efficacy and reducing systemic toxicity (1). Moreover, they also exhibit a good potential for surface modification and provide controlled and long-term release rates as well as prolonged bioactivity in the optimal dosage range, all of which result in decreased frequency of administration and increased patient compliance (2–4). However, to remain available for a sufficiently long period in the blood circulation, NPs must be able to escape from the clearance function of macrophages of the reticuloendothelial system (RES) located in the liver (Kupffer cells) and in the spleen (mononuclear phagocyte system, MPS) (5). In general, the proteins that are adsorbed from the blood (opsonins) onto the surface of the NPs activate a rapid uptake of the injected NPs by the MPS (6,7). Surface modification of systemic drug carriers by poly (ethylene glycol) (PEG) is one of the preferred ways to decrease opsonization by reducing interactions with blood proteins due to its hydrophilicity and steric repulsion effects (8). Doxil®/Caelyx® (PEGylated liposomal doxorubicin) is an example of a PEG-modified, long-circulating liposome that is currently used in clinical chemotherapy that provides greater anti-tumor activity and lower toxicity than doxorubicin solution (9).

Nevertheless, some recent reports indicate that repeated injections of PEGylated liposomes in the same animals at

certain intervals induce significant immune responses, resulting in a loss of the long circulation half-life of the liposomes (10–12). This effect is called the "accelerated blood clearance (ABC)" phenomenon in which the anti-PEG IgM, produced in the spleen after i.v. injection of PEGylated liposomes, selectively binds to PEG on the surface of the second dose of the PEGylated liposomes to cause rapid elimination and enhanced hepatic uptake of the subsequent doses (13). This effect poses a considerable clinical challenge because it decreases the therapeutic efficacy of an encapsulated drug after repeated administration and may cause adverse effects due to the altered tissue distribution of the drug. Accordingly, some studies have aimed to evaluate different structures that would be associated with less or no ABC. In one attempt, Xu *et al.* prepared PEGylated liposomes using cleavable PEG-lipid derivatives (PEG-CHMC and PEG-CHEMS) that showed a reduced or eliminated ABC effect after repeated injection in Wistar rats (14). In another study, Maitani and coworkers showed that gadolinium-containing PEG-poly(L-lysine)-based polymeric micelles did not induce ABC following their pre-administration at various doses and time intervals, whereas gadolinium liposomes induced the phenomenon. They concluded that the hydrophobic core of the micelle or the lipid bilayer of the PEGylated liposome has a major effect on this phenomenon (15). In addition to the carrier structure, the drug associated with the carrier also seems to be an important factor that may affect the ABC phenomenon. Laverman *et al.* found that repeated injections of Caelyx® did not induce the ABC phenomenon in a murine model (11). Gabizon *et al.* reported a reduced clearance of PEGylated liposomal doxorubicin after repeated injections in a clinical application, which was attributed to the toxic activity of the encapsulated doxorubicin against the RES (16). Ishida *et al.* demonstrated that, due to its interference with the proliferation of B cells, encapsulation of doxorubicin within liposomes may reduce the production of anti-PEG IgM and thus abrogate the immune response against PEGylated liposomes (17). Cui *et al.* also reported that PEGylated mitoxantrone liposomes did not induce the ABC phenomenon (18). These results suggest that repeated injections of liposomes loaded with cytotoxic drugs do not induce the ABC phenomenon. However, Deng and coworkers (19) recently showed that, unlike doxorubicin and mitoxantrone, repeated injections of PEGylated liposomal topotecan could still induce a strong ABC phenomenon in rats. They suggested that topotecan is a cell cycle phase-specific drug that can only inhibit a fraction of B lympholeukocytes in the S phase of the cell cycle. Accordingly, in comparison to cell cycle-nonspecific drugs, the toxic effect of topotecan on B cells may be reduced dramatically, with the result that a first injection of topotecan liposomes can still induce a strong ABC phenomenon for the second dose.

These findings suggest that anticancer drugs loaded within liposomes, based on their roles in different stages of cell proliferation, may induce different levels of ABC, although this hypothesis requires further investigation.

Notably, ABC studies have focused mainly on PEGylated liposomes, and very few studies have been reported on the pharmacokinetic behavior of polymeric NPs after their repeated injections in spite of the growing popularity of these nanostructures in drug delivery (20,21). To the best of our knowledge, the induction of the ABC phenomenon by PLGA-PEG NPs, an FDA-approved and commonly used polymeric carrier, and particularly those encapsulating an anticancer drug, has not been investigated.

Therefore, in this study, we first investigated whether the ABC effect is caused by repeated injections of PLGA-PEG NPs at various time intervals and polymer doses (ranging from 0.1 to 30 mg). We then studied the effect of etoposide, as a potent and cell cycle phase-specific anticancer drug, on ABC induction after two and three injections of PLGA-PEG NPs. In addition, we examined the production of the anti-PEG IgM antibody as a potential reason for the ABC phenomenon.

MATERIALS AND METHODS

Materials

Poly (lactic-co-glycolic acid)-poly (ethylene glycol) (PLGA-PEG) 5% (RGP d 5055) was obtained from Boehringer Ingelheim (Ingelheim, Germany). Etoposide was kindly provided by Cipla (Mumbai, India). Analytical-grade dichloromethane was purchased from Merck (Darmstadt, Germany). HPLC-grade acetonitrile and methanol were obtained from Caledon (Georgetown, Canada). All other chemical reagents used were of pharmaceutical grade.

Preparation and Characterization of Etoposide Loaded NPs

NPs were prepared by single-emulsion solvent evaporation method. Briefly, an exact amount of PLGA–PEG (45 mg) and etoposide (7.5 mg) were dissolved in DCM, This organic phase was added to the stirred aqueous solution containing an emulsifier and sonicated simultaneously with a sonicator (UP200S, Hielscher GmbH, Teltow, Germany) for about 2 min to form O/W single emulsion. The resulting NPs suspension was allowed to stir uncovered at 400 rpm for 4 h at room temperature until complete evaporation of the organic solvent. The freshly formed NPs were then separated by centrifugation for 20 min at 13000×g and 4°C (Optima L-90 K Beckman Coulter, Fullerton, CA, USA) and washed three times with distilled water, in order to gradually remove free drug and excess surfactant completely.

Drug content was measured in triplicate using a validated HPLC (Knauer, Germany) method. The HPLC system consisted of a Wellchrom K-1001 solvent delivery equipped with a model K-2700 diode array UV/VIS at 240 nm (Knauer, Germany). Analyses were carried out at ambient temperature on a Chromolith Performance RP-18e (100 mm × 4.6 mm i.d., Merck) coupled with a Chromolith RP-18e guard cartridge (5.0 mm × 4.6 mm i.d., Merck). The mobile phase was a mixture of acetonitrile and water (28:72, *v/v*) with a flow rate of 0.7 ml/min. The calibration curves were linear over the concentration range of 0.05–10 μg/ml and the coefficients of variation (CV) were all within 6%. Weighed amount of NPs was dissolved in acetonitrile by vortex agitation to get a clear solution and injected to HPLC via auto-injector after proper dilution with mobile phase. The encapsulation efficiency (% EE) and drug loading were calculated based on the following equations:

$$\%\mathrm{EE} = \left(\frac{\text{Amount of drug entrapped in NPs}}{\text{Initial amount of drug added}}\right) \times 100$$

$$\%\text{Drug loading} = \left(\frac{\text{Amount of drug entrapped in NPs}}{\text{Total amount of NPs}}\right) \times 100$$

The particle size, size polydispersity and zeta potential of the NPs were determined by dynamic light scattering, using a Malvern Nano ZS (Malvern Instruments, Worcestershire, UK). The instrument was calibrated with standard latex NPs (Malvern Instruments, Worcestershire, UK). Experimental values were the average of 3 different formulations.

Animal Experiments

Male Wistar rats (250 ± 10 g) were from the Pasteur Institute of Iran (Tehran, Iran). Each rat was housed in a cage with a 12 h light/12 h dark cycle at ambient temperature (22°C) and relative humidity of 55 ± 5%. The rats were fasted overnight before experimentation and had access to water *ad libitum*. All protocols and procedures were approved by the local ethics committee for animal experiments of Shahid Beheshti University of Medical Sciences (Tehran, Iran).

Male Wistar rats were randomly divided into 14 groups, and formulations were injected intravenously via the rat tail vein ($n = 6$ rats for each group). A dispersion of NPs was freshly prepared and diluted in normal saline immediately before administration. Control animals received an injection of normal saline as the first dose instead of NPs. The injection schemes for the NPs are presented in Table I. Blood samples (250 μl) were taken from the tail vein into heparinized microcentrifuge tubes immediately prior to (blank sample) and following the test dose at the designated time intervals for up to 24 h. After each sampling, 250 μl of normal saline was administered to prevent changes in the central compartment volume and electrolytes. Samples were centrifuged at 2500 × g for 5 min, and plasma was subsequently separated and stored at −20°C until analysis. Drug concentration in plasma was analyzed using the HPLC method developed in our laboratory (22). The analytical method was linear in the concentration range of 0.04–10 μg/ml, with a limit of quantification and limit of detection of at least 0.04 and 0.02 μg/ml, respectively.

Pharmacokinetic analysis was performed by using noncompartmental method (23). The elimination rate constant (K) was estimated from the least-square regression of plasma concentration-time data points in the terminal log-linear region of the curves. The elimination half-life ($t_{1/2}$) was calculated as 0.693 divided by K. The area under the plasma concentration-*versus*-time curve from time zero to the last measurable time ($AUC_{0\text{-last}}$) was calculated using the trapezoidal rule. The area from the last time to the infinity ($AUC_{\text{last-}\infty}$) was estimated by dividing the last measurable plasma concentration by K. The total AUC ($AUC_{0\text{-}\infty}$, hereafter referred to as AUC) was obtained by addition of $AUC_{0\text{- last}}$ to the $AUC_{\text{last-}\infty}$. It is worth to mention that the ratio of $AUC_{0\text{-last}}$ to total AUC was more than 90% in all calculations. The clearance (Cl) was calculated by dividing dose by AUC.

Determination of Anti-PEG IgM Antibody Levels

Quantification of the IgM antibody reactive to PEG-PLGA in plasma was determined using a slightly modified procedure based on a published ELISA method (21). Briefly, PEG-PLGA (10 nmol) in 50 μl acetonitrile was added to a 96-well Maxisorb ELISA plate (Nunc, Roskilde, Denmark) and air-dried completely for 1 h (all incubations were carried out at room temperature). Then, 200 μl of blocking buffer (50 mM Tris–HCl (pH 8.0), 0.14 M NaCl and 1% BSA) was added to each well and incubated for 1 h. After incubation, the wells were washed three times with washing buffer (50 mM Tris/HCl (pH 8.0), 0.14 M NaCl and 0.05% Tween 20), and 100 μl of serum samples were diluted appropriately and added to the wells, followed by incubation for 1 h and washing five times with the washing buffer. Horseradish peroxidase (HRP)-conjugated antibody (100 μl of 0.2 μg/ml, goat anti-rat IgM IgG-HRP conjugate, Bethyl Laboratories, Inc., TX, USA) in blocking buffer containing 0.05% Tween 20 was added to each well. After incubation for 1 h, the wells were washed five times with washing buffer. Staining was carried out by a 5 min incubation with 100 μl of *o*-phenylenediamine (1 mg/ml, Sigma–Aldrich, MO, USA) and stopped by adding 100 μl of 2 N H_2SO_4. The absorbance was measured at 490 nm using a Stat Fax-2100 microplate reader (Awareness Technology Inc., CA, USA).

Table I The Injection Protocols

Group	First injection characteristics			Time interval between doses (days)	Second injection	Third injection
	injected NPs for the first dose	Polymer dose (mg)	drug dose (mg/kg)			
Control	No	0	0	0	ET-high[a]	No
I-3	Empty PLGA-PEG NPs	0.1	0	3	ET-high	No
I-5	Empty PLGA-PEG NPs	0.1	0	5	ET-high	No
I-7	Empty PLGA-PEG NPs	0.1	0	7	ET-high	No
I-14	Empty PLGA-PEG NPs	0.1	0	14	ET-high	No
I-28	Empty PLGA-PEG NPs	0.1	0	28	ET-high	No
D-0.1[b]	Empty PLGA-PEG NPs	0.1	0	7	ET-high	No
D-1	Empty PLGA-PEG NPs	1	0	7	ET-high	No
D-10	Empty PLGA-PEG NPs	10	0	7	ET-high	No
D-20	Empty PLGA-PEG NPs	20	0	7	ET-high	No
D-30	Empty PLGA-PEG NPs	30	0	7	ET-high	No
ET-low	Etoposide loaded PLGA-PEG NPs	0.1	0.04	7	ET-high	No
ET-high	Etoposide loaded PLGA-PEG NPs	20	8	7	ET-high	No
PEG-negative	Empty PLGA NPs	20	0	7	ET-high	No
Dose3[c]	Etoposide loaded PLGA-PEG NPs	20	8	7	ET-high	ET-high

In each group $n = 6$ rats

[a] ET-high contains 8 mg/kg etoposide and 20 mg PLGA-PEG

[b] The results of D-0.1 and I-7 are the same

[c] time interval between the second and third doses is 7 days

Statistical Analysis

The results are expressed as the mean ± standard deviation and were analyzed with a one-way ANOVA using SPSS 17.0 software.

RESULTS

The Characteristics of NPs

NPs were prepared by the single emulsion-solvent evaporation method, and their characteristics are listed in Table II. The mean particle size for NPs was approximately 170 nm with a polydispersity value of approximately 0.1. The etoposide-loaded PLGA-PEG NPs were associated with a high encapsulation efficiency (approximately 90%) and showed a good sustained release profile of the drug (more than a week).

Induction of the ABC Phenomenon

Previous studies on PEGylated liposomes have demonstrated that the time interval between the liposome injections is a key factor to elicit ABC and to the extent of this phenomenon that is induced following prior injection of carriers (11). Therefore, the first step of the present study was to find the time interval that resulted in the greatest increase in ABC of the PEGylated PLGA NPs. Rats were divided into five groups (I-3, I-5, I-7, I-14 and I-28) and were injected with etoposide-containing NPs as the second dose (8 mg/kg etoposide) after various time intervals after the first injection (3, 5, 7, 14 and 28 days, respectively). Based on the pilot study, a low polymer dose of empty PLGA-PEG NPs (0.1 mg) was applied as the first dose to exclude the effect of the polymer dose on ABC. The control group received a single i.v. dose of etoposide-containing PLGA-PEG NPs (8 mg/kg etoposide).

The log plasma concentration-*versus*-time curves after the second doses following different time intervals are shown in Fig. 1; the profile in the absence of a prior injection is also shown as a control. As observed in the 7-day time interval

Table II NPs Characteristics for i.v. Injection to Rats

NPs	Size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
PLGA-PEG	168 ± 2	0.08	−34.30 ± 0.41	NA
Etoposide loaded PLGA-PEG	173 ± 3	0.07	−32.20 ± 0.61	89.7 ± 5.1
PLGA	177 ± 5	0.10	−23.90 ± 0.76	NA

NA not applicable; Data is presented as mean ± SD, $n = 3$

group (I-7), the second dose was cleared more rapidly from circulation compared to the control and other groups. After 2 h, the concentration in the I-7 group was approximately 1/9 of the control (816±168 of I-7 *versus* 6878±1715 ng/ml of control, $p<0.00001$). At 6 h, the concentration in I-7 remained the lowest, followed by I-5, with both being significantly lower than that of the control (52±16 of I-7 and 116±42 ng/ml of I-5 *versus* 1507±822 ng/ml for control; $P<0.005$). When the time interval for injection was extended to 28 days, the plasma concentration – time profile of the second dose was similar to that of the control. Pharmacokinetic parameters were also calculated and are shown in Table III. As shown, the AUC value gradually decreased when the time interval increased from 3 to 7 days. In the I-7 group, the AUC reached the minimum level (640±136 μgmin ml^{-1} *versus* the control with 3743±1344 μgmin ml^{-1}, $p<0.001$), and the clearance was the highest (3.2±0.6 mlmin^{-1} *versus* the control with 0.6±0.2 mlmin^{-1}, $p<0.00001$). After 14 days, these parameters approached the control values, and the results of the I-28 group were comparable to those of the control. These results showed that, similar to PEGylated liposomes, repeated injections of PLGA-PEG NPs in rats induced the ABC phenomenon and that the level of induction was strongly dependent on the time interval between the doses. As the greatest increase in ABC induction was observed with a time interval of 7 days between two NP doses, all subsequent experiments were performed using a 7-day time interval.

Effect on ABC of the Polymer Dose in the First Injection of PLGA-PEG NPs

To evaluate the effect of the polymer dose in the first injection on ABC induction, doses of 0.1, 1, 10, 20 and 30 mg of polymer were used for the groups of rats as follows: D-0.1, D-1, D-10, D-20 and D-30 groups (Table I). It is important to mention here that the results from the I-7 group with 0.1 mg polymer were considered to be the results for D-0.1. Figure 2 shows the log plasma concentration-time curve after the test dose in different groups in comparison to the control.

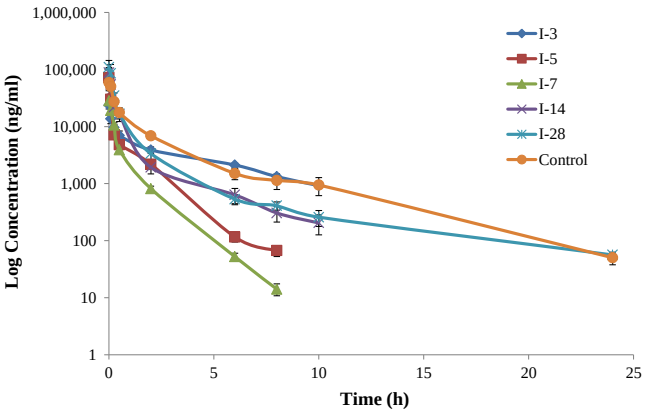

Fig. 1 Log plasma concentration (mean ± S.E.) *vs.* time profiles of etoposide after the test dose administered following various time intervals after the first injection of empty PLGA-PEG NPs (0.1 mg). The control group received a single i.v. dose of etoposide-containing PLGA-PEG NPs (8 mg/kg etoposide).

Increasing the polymer dose from 0.1 to 20 mg did not significantly affect the profile at any time point. Further increasing the polymer dose to 30 mg caused a considerable increase in plasma concentration that was significantly higher than even the control group (*e.g.*, at 2 h, 166618±4728 *vs.* control with 6878±1715 μgml^{-1}, $p<0.00005$, and at 6 h, 8938±4613 *vs.* 1507±822 μgml^{-1} in control, $p<0.0005$). These results are in agreement with the pharmacokinetic data (Table III). For the D-30 group, which received a higher polymer dose in the first injection, the AUC value (12644±5126 μgmin ml^{-1}) for the test dose (second dose) was distinctly higher ($p<0.0005$), and the clearance (0.2±0.1 mlmin^{-1}) was lower than that of the control ($p<0.01$). These results clearly indicate that an effective conversion of ABC has occurred by using a high polymer dose as the pretreatment injection.

Effect on ABC of PEG in the First Injection

To evaluate the effect of the presence of PEG on ABC induction in rats, NPs were prepared with the PLGA matrix, a dose of 20 mg polymer was injected as the first dose, and the test dose was injected after 7 days (referred to as the PEG-negative group in Table I). Log plasma concentration-*versus*-time curves after the test dose in PEG-negative and control groups are shown in Fig. 3. No significant differences were found in the plasma concentrations of the two groups at 2 (8291±2524 *versus* 6878±1715 μgml^{-1}, $p>0.05$) and 6 h (1861±663 *versus* 1507±822 μgml^{-1}, $p>0.05$) after the test dose injection. In addition, the AUC obtained for the PEG-negative group was 5677±1042 μgmin ml^{-1}, and the clearance was 0.7±0.1 mlmin^{-1}, neither of which was significantly different from the corresponding control values ($p>0.05$). These results reveal that the two groups of control and PEG-negative are quite comparable in terms of pharmacokinetic parameters with no ABC effect, which confirms the vital role played by the PEG coating of injected NPs in ABC induction.

Effect on ABC of the Presence of Etoposide in the Initial Dose of PLGA-PEG NPs

The effect of etoposide, a cytotoxic drug, on the induction of ABC was evaluated by the pre-administration of PLGA-PEG NPs containing low (0.04 mg/kg) and high doses (8 mg/kg) of etoposide to rats (groups ET-low and ET-high, respectively, Table I). The results were compared to the control and to the groups that were pretreated with the same dose of polymer as empty NPs (groups of D-0.1 and D-20). The log plasma concentration-time curves, after administration of the test

Table III Pharmacokinetic Parameters of Etoposide after the Last Injection of Test Dose in Different Groups

Group	AUC ($\mu g min ml^{-1}$)	p value[a]	Clearance ($ml min^{-1}$)	p value[a]	$t_{1/2}$ (min)	p value[a]
Control	3743.02 ± 1344.14		0.59 ± 0.21		208.77 ± 18.69	
I-3	2051.52 ± 372.58	> 0.05	1.00 ± 0.21	< 0.05	195.16 ± 16.34	> 0.05
I-5	1101.28 ± 273.86	< 0.01	1.90 ± 0.46	< 0.05	123.26 ± 16.81	< 0.05
I-7	639.56 ± 136.16	< 0.001	3.23 ± 0.63	< 0.00001	62.19 ± 8.94	< 0.00001
I-14	1977.91 ± 392.24	< 0.05	1.04 ± 0.21	< 0.05	147.07 ± 41.34	< 0.05
I-28	2822.94 ± 918.99	> 0.05	0.74 ± 0.25	> 0.05	252.52 ± 29.76	> 0.05
D-0.1	639.56 ± 136.16	< 0.001	3.23 ± 0.63	< 0.00001	62.19 ± 8.94	< 0.00001
D-1	623.72 ± 115.35	< 0.0001	3.29 ± 0.60	< 0.00001	71.29 ± 14.16	< 0.00001
D-10	667.74 ± 60.02	< 0.0001	3.02 ± 0.28	< 0.0001	84.07 ± 11.39	< 0.00001
D-20	849.55 ± 231.00	< 0.0001	2.49 ± 0.65	< 0.001	147.60 ± 29.94	< 0.01
D-30	12644.37 ± 5126.08	< 0.001	0.18 ± 0.06	< 0.01	330.67 ± 114.55	< 0.05
ET-low	884.82 ± 194.07	< 0.05	2.32 ± 0.45	< 0.05	106.05 ± 13.31	< 0.001
ET-high	1365.80 ± 456.86	< 0.01	1.63 ± 0.52	< 0.01	157.74 ± 52.54	> 0.05
PEG-negative	5676.97 ± 1042.43	> 0.05	0.36 ± 0.09	> 0.05	174.42 ± 42.50	> 0.05
Dose3	2847.04 ± 516.27	> 0.05	0.72 ± 0.12	> 0.05	199.97 ± 26.26	> 0.05

Data is presented as mean ± SD, n = 6

[a]Compared to the control

dose to the aforementioned three groups, are depicted in Fig. 4. As shown, plasma profiles for the ET-low and ET-high groups were quite comparable at various time points, but both profiles were significantly different from that of the control. Additionally, the pharmacokinetic parameters, such as AUC, Cl and $t_{1/2}$, for ET-low and ET-high were identical (Table III). However, these parameters were significantly different from the control ($p<0.05$). These results show that pretreatment with etoposide-loaded PLGA-PEG NPs induces the ABC effect and that the dose of the drug has no influence on ABC. To better understand the influence of etoposide as a cytotoxic drug on ABC induction, the pharmacokinetic data from the ET-low and ET-high groups were compared to those from D-0.1 and D-20, which were only different by drug incorporation. Comparison of the ET-low group with D-0.1 and of the ET-high group with D-20 did not reveal any significant differences in AUC, Cl, or $t_{1/2}$. These data suggest that drug incorporation did not affect the ABC phenomenon induced by PLGA-PEG NPs.

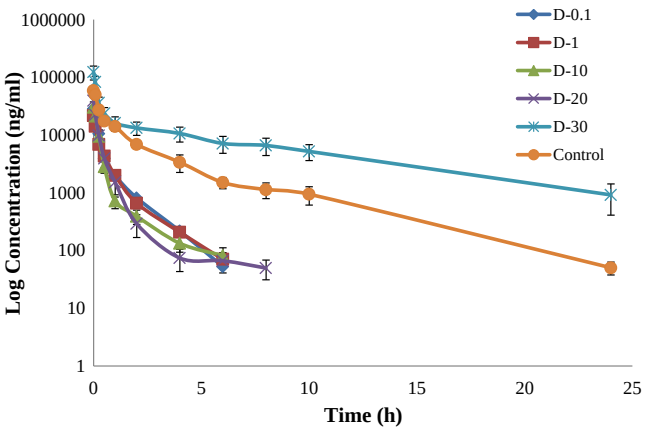

Fig. 2 Log plasma concentration (mean ± S.E.) *vs.* time profiles of etoposide after the test dose administered following the first injection of empty PLGA-PEG NPs with different polymer doses. Polymer doses of 0.1, 1, 10, 20 and 30 mg were used for the groups of rats as follows: D-0.1, D-1, D-10, D-20 and D-30 groups The control group received a single i.v. dose of etoposide-containing PLGA-PEG NPs (8 mg/kg etoposide).

Effect on ABC of the Third Dose of Etoposide-Loaded PLGA-PEG NPs

To investigate the effects of the injection of sequential doses (more than two doses) of drug-loaded PLGA-PEG NPs on the ABC phenomenon, three injections of ET-high were administered to a group of rats with a time interval of 7 days between the doses (referred to as group Dose 3 in Table III). The log

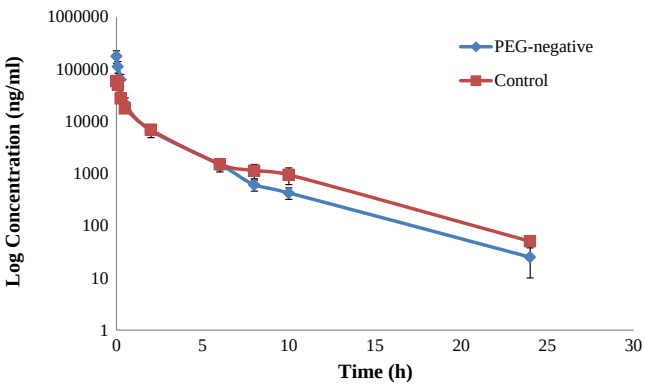

Fig. 3 Log plasma concentration (mean ± S.E.) *vs.* time profile of etoposide following the test dose pre-administered with PEG negative NPs. The control group received a single i.v. dose of etoposide-containing PLGA-PEG NPs (8 mg/kg etoposide).

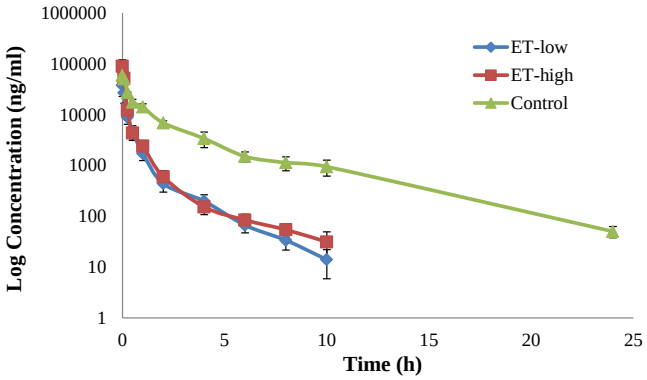

Fig. 4 Log plasma concentration (mean ± S.E.) *vs.* time profiles of etoposide following the test dose pre-administered with different doses of etoposide-loaded PLGA-PEG NPs in each group. The control group received a single i.v. dose of etoposide-containing PLGA-PEG NPs (8 mg/kg etoposide).

plasma concentration-time curve of etoposide after the last injection of the NPs has been shown in Fig. 5. The profile for the third dose group was obviously comparable to that of the control group, and this result was further confirmed by statistical analysis of the representative plasma concentrations at 2, 6 and 10 h after the administration of the NPs. At all of these time points, the concentration for the Dose 3 group was greater than that for ET-high ($p<0.05$), whereas the plasma concentrations of Dose 3 and the control were not significantly different for any of the time points, except for the 2 h time point ($p<0.01$). These results were confirmed by the AUC, Cl and $t_{1/2}$ data (Table III), which showed no significant differences when compared to the control ($p>0.05$). However, when pharmacokinetic parameters following the third dose (Dose 3) were compared to those measured after the second dose (ET-high), it was found that the AUC and $t_{1/2}$ values were higher ($p<0.01$) and that Cl was lower for the Dose 3 group in comparison to the ET-high group.

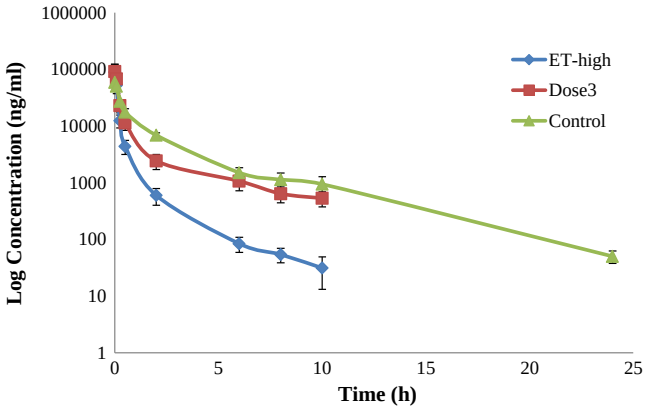

Fig. 5 Log plasma concentration (mean ± S.E.) *vs.* time profiles of etoposide following the test dose pre-administered with one (group ET-high) or two (group Dose 3) doses of etoposide-loaded PLGA-PEG NPs. The control group received a single i.v. dose of etoposide-containing PLGA-PEG NPs (8 mg/kg etoposide).

IgM Determination in Rats Following Intravenous Injection of NPs

The anti-PEG IgM antibody level was determined in each group by using the ELISA method. Figure 6 shows the results and the significant differences in animals that received the second dose following various time intervals (groups I-3 to I-28) in comparison to the control. The highest IgM level was clearly observed in the rats of the I-7 group that received the test dose after 7 days, which was in agreement with a higher ABC induction in this group. By keeping the time interval at 7 days while increasing the polymer dose from 0.1 to 20 mg (D-0.1 to D-20, Fig 6), the IgM reached a much higher level. Further increase of the polymer dose to 30 mg did not affect the IgM level. With regard to the effect of etoposide on IgM production, our results indicated that the IgM levels in rats pretreated with etoposide-loaded NPs (in ET-low and ET-high groups) were the same as those in rats pretreated with the corresponding empty NPs (D-0.1 and D-20, respectively). In addition to confirming the effect of the polymer dose on the antibody levels, these results could exclude the interference of etoposide from antibody production. To further evaluate the role of PEG, IgM levels were also determined in animals that received PLGA-NPs as the first dose, and these levels were found to be the same as that of the control animals. A high level of the antibody was found in Dose 3 in which IgM was produced after sequential doses of drug-loaded PLGA-PEG NPs, whereas the intensity of the ABC phenomenon showed the opposite trend (Fig. 5).

DISCUSSION

PEGylated nanocarriers represent promising approaches to improve the pharmacokinetic characteristics of encapsulated drugs and to enhance their circulation half-life for better

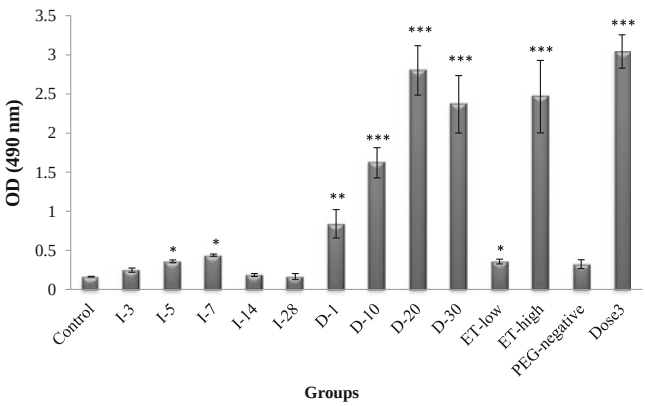

Fig. 6 Anti-PEG IgM response in each group of rats just before the test dose injection and in the control group (without NP injection). Values represented as the mean ± S. E. of 6 serum samples. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to the control.

therapeutic efficacy (24). PEG is assumed to provide a steric barrier around the NPs because of its hydrophilicity that prevents the adsorption of opsonins onto the surfaces of the NPs and reduces particle uptake by MPS as the major elimination route of NPs (25). However, the ABC phenomenon, which has been reported for some PEGylated nanostructures, may pose challenges to the clinical application of formulations that require repeated administration. This effect and possible underlying mechanisms have been extensively evaluated for liposomes (26,27).

It is proposed that the PEG polymer is known as a highly repetitive structure resembling a class-2 thymus- independent antigen. Once the PEGylated liposomes reach the spleen after the first injection, they bind to surface immunoglobulins on reactive B-cells in the splenic marginal zone and consequently the production of anti-PEG IgM antibody is triggered that is independent of T cell help (17,28). When the second dose of PEGylated liposomes was injected the secreted IgM selectively attach to the PEG on these liposomes. This, in turn, leads to complement activation and opsonization of the liposomes by C3 fragments and, consequently, to enhanced uptake of the liposomes by the Kupffer cells in liver (29). However, recent investigations have indicated that the structure and the contents of NPs are crucial factors that could reduce or even abolish the ABC phenomenon.

PLGA–PEG is an FDA-approved and commonly used polymeric drug carrier (2); however, to the best of our knowledge, there has been no report on the repeated injections and ABC of this carrier. In this study, we evaluated ABC induction by PEGylated PLGA NPs. The effects of encapsulated etoposide as a chemotherapeutic agent, the doses of the polymer and the drug and the injection of a third dose on the ABC phenomenon were also studied.

The results demonstrated ABC induction by PLGA-PEG NPs in a time-dependent manner. Based on a pilot study, a low dose of 0.1 mg polymer was pre-administered intravenously to five groups of rats for the first step. The test doses were injected after 3, 5, 7, 14 and 28 days in each group (I-3 to I-28 groups). The results showed that a 7-day interval between doses was the time period that triggered ABC most effectively (Fig. 1). In addition, the anti-PEG IgM level in this group was the highest among the aforementioned five groups. This finding confirms previous reports that the production of IgM directed against the PEG coating could be considered as the most likely mechanism for the enhanced clearance of the second injection (28,30) (Fig. 6). Dams *et al.* investigated the induction of blood clearance in rats, mice and rhesus monkeys, and they found that the ABC phenomenon is observed in rats and rhesus monkeys, but not in mice (10). In 2003, Ishida *et al.* (31) reported the same phenomenon in mice, although they found accelerated elimination with a time interval of 10 days between doses, which was different from the 7-day interval for rats. Our findings also confirm 7 days as the optimal time interval for the most effective ABC induction in the rat, which is the most common model for *in vivo* studies. As the time interval for triggering the ABC effect is dependent on the animal species, determination of this time interval in humans could be considered to be an important clinical issue. Despite ABC triggering by even a low dose of PLGA-PEG copolymer, drug-loaded NPs are usually applied in higher polymer doses. Thus, it is necessary to clarify the effect of the polymer dose on ABC induction. A set of experiments was performed in four groups of rats in which pre-administered NPs were injected in doses of 1, 10, 20 and 30 mg (D-1, D-10, D-20 and D-30 groups). Data from I-7 rats administered a 0.1 mg polymer dose was considered as D-0.1 (Table I). The pharmacokinetic parameters (Table III) and the plasma concentration *vs.* time profiles (Fig. 2) demonstrated comparable induction of ABC using 0.1 to 20 mg of polymer, whereas a further increase in the polymer dose up to 30 mg significantly abolished the ABC and led to decreased clearance, which was not only lower than that of the D-0.1 group (by 18.5 fold) but also lower than that of the control (by 3.4 fold). These results indicated that the elimination rate of NPs is strongly affected by the highest PLGA-PEG dose, most likely because of saturation of the MPS (32). In liposome experiments, Laverman *et al.* (11) found an inverse correlation between the lipid dose and ABC induction. Although their finding was confirmed by other investigators for stealth liposomes (26), Ishihara *et al.* (21) reported that the induction of the ABC phenomenon by PLGA-PEG NPs was not clearly affected by the initial dose. In the present study, we demonstrated that a specific range of the polymer dose did not affect ABC induction, but that a higher dose of polymer clearly suppressed the ABC phenomenon. As various doses of polymeric structures are generally used in clinical applications, the effect of the polymer dose on the clearance of NPs and ABC induction should be evaluated for every individual nanostructure and in a wide range of doses.

We also tested the effect of polymer dose on the serum level of anti-PEG IgM. There are few reports on the dose–response effect on the anti-PEG IgM response. Lu *et al.* showed that successive high doses of 30 mg/kg/day of cationic bovine serum albumin (CBSA) NPs could not produce CBSA-specific IgM in mouse serum and did not display any ABC phenomenon (33). In another study, Tagami *et al.* reported an inverse relationship between the dose of a PEG-coated siRNA lipoplex and anti-PEG IgM production in mice (34). In contrast, Taguchi found that the IgM response against PEGylated hemoglobin vesicles (HbV) was dose-dependent in mice as the degree of IgM elevation at a high dose of HbV was significantly larger than that for a low dose (35). Different results were obtained by Ma *et al.* who found no clear relationship between the anti-PEG IgM levels and the phospholipid dose (19). In this study, we observed that by increasing the polymer dose from 0.1 to 20 mg, the level of IgM increased almost

proportionally (Fig 6), although the degree of ABC induction was not affected by the polymer dose or by the IgM level, remaining somewhat constant in the mentioned range of polymer dose (Fig 2 and Table III). These results show that a threshold of IgM level is required for triggering MPS and the enhanced clearance of NPs, as proposed by Ishida *et al.* (17). However, higher antibody levels do not apparently elevate the immune response. This finding may be explained by the capacity of macrophages for phagocytosis being limited by the number of the available phagocytic cells such that increasing the IgM levels by increasing the polymer dose (Fig 6) will not change the magnitude of the clearance of the second dose (approximately 3 $ml min^{-1}$, Table III). A further increase in the polymer dose from 20 to 30 mg did not significantly affect IgM levels (Fig. 3), but as it is clear from Fig. 2 and Table 3, the higher polymer dose (30 mg, D-30 group, Fig. 2, Table 3) likely due to saturation of phagocytic cells, resulted in an abrogation of ABC induction and higher values for AUC and half-life of NPs in the relevant group (D-30) when compared to those for the control (AUC = 12644.37 and 3743.02 $\mu g min\ ml^{-1}$ and $t_{1/2} = 330.67$ and 208.77 min, respectively; $p < 0.05$).

The data from the PEG-negative group, in which a relatively high dose of non-PEGylated PLGA NPs was pre-administered, verified the fundamental role of PEG for ABC induction. This finding was not consistent with those of some previous studies performed for liposomes, which indicated that the PEG coating was not necessarily required for ABC induction (10,11,36). In addition, as shown in Fig 6, the IgM level was not different from that observed in the control group. This result is not in agreement with those reported by Kiwada and co-workers (29) who indicated that conventional liposomes, despite the lack of a PEG-coating, could produce a significant level of anti-PEG IgM. They suggested that the antigenic epitope capable of generating anti-PEG IgM is present not only in the PEG moiety but is also displayed in other components of the liposome or on the liposomal structure. Taken together, our results indicate that, unlike liposomes, the PEG moiety in PLGA-PEG NPs does play an essential role in the induction of ABC effect and also in the production of anti-PEG IgM.

As the other main purpose of our studies, we investigated the effect of etoposide, a commonly used anticancer drug, encapsulated in PLGA-PEG NPs on ABC induction. Long-circulating polymeric NPs are frequently investigated for cancer drug delivery because of the EPR (enhanced permeability and retention) effect. However, the induction of the immune response upon repeated administration of nanocarriers may present a tremendous challenge to their clinical use, as multiple injections of nanostructures such as liposomes are very common in clinical settings. It is noteworthy that, in contrast to empty PEG-liposomes, doxorubicin-loaded liposomes have not been reported to induce the ABC phenomenon (11,17). The most likely reasons proposed were based on the drug toxicity for RES and the reduced production of anti-PEG IgM caused by the interference of doxorubicin with the proliferation of B cells. However, Deng and coworkers have recently reported that a pre-injection of PEGylated liposomal topotecan still produced a strong ABC effect (19). They assumed that topotecan was a cell-cycle-specific drug, which could only inhibit the population of B cells in the splenic marginal zone occupying the S phase, and that its lipophilicity and unsatisfactory retention inside liposomes might be reasons for the observed ABC effect.

These data show that the characteristics of a drug that is loaded into nanocarriers may significantly influence the pharmacokinetic parameters after repeated doses. In this study, we found a distinct ABC phenomenon with etoposide-loaded PLGA-PEG NPs when injected as an initial dose (Fig. 4). The drug dose studies indicated that the magnitude of ABC induction as well as the anti-PEG IgM levels in rats pre-treated with drug-loaded PLGA-PEG NPs (groups of ET-low and ET-high) were equal to those in rats pre-treated with empty NPs (groups of D-0.1 and D-20). The negligible influence of encapsulated etoposide on ABC shows that, when compared to empty NPs, the uptake of drug-loaded NPs by MPS did not have any marked effect on their clearance function. This finding suggests that unlike liposomal doxorubicin, the encapsulated etoposide seems to have a minor toxic effect on B cells and macrophages, thus inducing a strong ABC effect by repeated injection of the drug-loaded PLGA-PEG nanoparticles. This finding supports the mechanism that has recently been proposed by Deng and coworkers for topotecan liposomes (19). Etoposide, like topotecan, is a cell-cycle-specific drug (37) and is expected to only affect the B cells that are in the G2/M phase. Thus, drug-loaded NPs cannot inhibit the entire B cell population of cells in the splenic marginal zone, which could also be confirmed by the production of a high level of IgM, leading to the appearance of an ABC effect (19). In addition to this explanation, Deng and coworkers proposed the unsatisfactory retention of topotecan within liposomes due to its lipophilicity (plasma topotecan was detectable for up to only 4 h) as another factor that could influence the drug effects on B cells or macrophages. In the present study, we were able to measure etoposide concentrations in the plasma for up to 24 h, which demonstrates appropriate *in vivo* stability of the formulated PLGA-PEG NPs. Therefore, the specificity of the drug for particular phase(s) of the cell cycle may be a plausible reason, although the exact mechanism requires further investigation.

Another objective of our investigations was the evaluation of the ABC phenomenon after three sequential doses of etoposide-loaded PLGA-PEG NPs (Group Dose 3, Fig. 5 and Table III). Neither the pharmacokinetic parameters nor the plasma-concentration time curves obtained from Dose 3

showed any ABC effect; while antibody level remained high for all of the groups (Fig. 6). The high IgM level shows the proper functioning of the B cells for antibody production. Therefore, the extended circulation time of the third dose of the NPs might be the result of MPS saturation and reduced NP uptake due to the accumulation of polymer after repeated administration. Our results for the levels of the antibody and ABC induction after the third dose are not in accordance with the previous studies that have investigated the ABC phenomenon after more than two sequential doses. Dams and coworkers indicated that the ABC phenomenon was less pronounced after the third and fourth doses in rats that received weekly injections of empty PEGylated liposomes. However, according to their findings, the ABC after the second dose was not Ig-mediated (10). In contrast, a high level of anti-PEG IgM was detected in our study. However, Ishihara *et al.* showed that ABC is still induced by sequential administration of PEG-PLA NPs (0.05 and 1 mg) containing prostaglandin E1. They found an inverse correlation between the dose frequency and detected IgM levels (38), which was attributed to the apoptosis and anergy of immune cells in response to cumulative amounts of polymer or encapsulated drug, an explanation that was also proposed by Ishida *et al.* for liposomes (18). In contrast, we found that the antibody levels produced after the second and third doses (20 mg of polymer) were similarly high ($p > 0.05$).

CONCLUSIONS

PEGylated PLGA NPs, a commonly used, long-circulating polymeric nanocarrier, showed an obvious ABC phenomenon 7 days after the pre-administration of empty PLGA-PEG NPs with a low polymer dose. Increasing the first injection polymer dose within the range of 0.1 to 20 mg did not alter the intensity of ABC induction, whereas a clear positive correlation was observed between the serum IgM level and the polymer dose. Further increase of the polymer dose to 30 mg led to enhanced circulation time of the test dose beyond that of the control, whereas the IgM levels plateaued. The critical role of PEG coating in the first injection was verified by the pre-administration of empty non-PEGylated PLGA NPs, which did not change the pharmacokinetic characteristics of the test dose compared to the control; in addition, no anti-PEG IgM was detected. The ABC phenomenon was examined following the injection of etoposide-loaded PLGA-PEG in two and three sequential doses. The results indicated that pre-injection of encapsulated etoposide in different doses still induced the ABC phenomenon for the second dose. The third dose did not demonstrate ABC any longer, although the antibody level remained high.

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