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Drug Delivery

Development and characterization of hyaluronic acid–anchored PLGA nanoparticulate carriers of doxorubicin

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Abstract A novel hyaluronic acid-poly(ethylene glycol)-poly(lactide-co-glycolide) (HA-PEG-PLGA) copolymer was synthesized and characterized by infrared and nuclear magnetic resonance spectroscopy. The nanoparticles of doxorubicin (DOX)-loaded HA-PEG-PLGA were prepared and compared with monomethoxy(polyethylene glycol) (MPEG)-PLGA nanoparticles. Nanoparticles were prepared using drug-to-polymer ratios of 1:1 to 1:3. Drug-to-polymer ratio of 1:1 is considered the optimum formulation on the basis of low particle size and high entrapment efficiency. The optimized nanoparticles were characterized for morphology, particle size measurements, differential scanning calorimetry, x-ray diffractometer measurement, drug content, hemolytic toxicity, subacute toxicity, and in vitro DOX release. The in vitro DOX release study was performed at pH 7.4 using a dialysis membrane. HA-PEG-PLGA nanoparticles were able to sustain the release for up to 15 days. The tissue distribution studies were performed with DOX-loaded HA-PEG-PLGA and MPEG-PLGA nanoparticles after intravenous (IV) injection in Ehrlich ascites tumor–bearing mice. The tissue distribution studies showed a higher concentration of DOX in the tumor as compared with MPEG-PLGA nanoparticles. The in vivo tumor inhibition study was also performed after IV injection of DOX-loaded HA-PEG-PLGA nanoparticles up to 15 days. DOX-loaded HA-PEG-PLGA nanoparticles were able to deliver a higher amount of DOX as compared with MPEG-PLGA nanoparticles. The DOX-loaded HA-PEG-PLGA nanoparticles reduced tumor volume significantly as compared with MPEG-PLGA nanoparticles.

Key words: Hyaluronic acid; Nanoparticles; Doxorubicin; Tumor Targeting

Introduction

Cancer is the second leading cause of death in the developed world, killing over 500,000 people in the United States in 2000 [1]. Chemotherapy is a major therapeutic approach for the treatment of localized and metastasized cancers. The selective increase in tumor tissue uptake of anticancer agents would be of great interest in cancer chemotherapy, because anticancer drugs are not specific to cancer cells. Routes of administration, biodistribution, and elimination of available chemotherapeutic agents can be modified by drug delivery systems to optimize drug therapy. Conventional cancer therapy and diagnostics involve the application of catheters, surgery, biopsy, chemotherapy, and radiation. Most current anticancer agents do not greatly differentiate between cancerous and normal cells. This leads to systemic toxicity and adverse effects. Consequently, the systemic application of these drugs often causes severe side effects in other tissues (e.g., bone marrow suppression, cardiomyopathy, and neurotoxicity), which greatly limits the maximal allowable dose of the drug. In addition, rapid elimination and widespread distribution into nontargeted organs and tissues requires the administration of a drug in large quantities, which is uneconomical and is often complicated because of nonspecific toxicity. Nano-

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technology could offer a less invasive alternative, enhancing the life expectancy and quality of life of the individual with cancer. The diameter of human cells spans 10 to 20 μm. The size of cell organelles ranges from a few nanometers to a few hundred nanometers. Nanoscale devices can readily interact with biomolecules on the cell surface and within the cells in a noninvasive manner, leaving the behavior and biochemical properties of those molecules intact [2].

Nanoparticles are colloidal particles that are less than 1 μm in diameter. In drug delivery applications nanoparticles have several merits, such as ease of purification and sterilization, drug targeting possibility, and a sustained-release action [3]. Targeted drug delivery systems can optimize the therapeutic index of antitumor drugs by increasing the drug concentration ratio of diseased tissue to normal tissue. In recent years interest has grown enormously in the identification of ligands that able to recognize specific cancer cells as target sites [4].

The main disadvantage with drug targeting to other sites in the human body is the rapid uptake of intravenously injected particulate drug carriers by the mononuclear phagocyte system. The most popular polymer material to modify particulate surfaces so as to avoid recognition by cells of the mononuclear phagocyte system is poly(ethylene glycol) (PEG). In general, biodegradability is an important requirement for the application of colloidal carriers in humans. Therefore, PEG-modified biodegradable drug carriers were produced by conjugating PEG chains covalently to their surface so as to avoid the displacement of adsorbed copolymers such as poloxamer by various blood components. In this study nanoparticles were prepared using di-block PEG-poly(lactide) (PLA) or blends of PLA and PEG-PLA polymers, where the PEG average molar mass was 2000 or 5000 g/mole. These particles showed a distinctly increased blood half-life as compared with PLA particles surface modified by poloxamer 188 [5]. Nanoparticles of MPEG-poly(ε-caprolactone) (PCL) and MPEG-PLGA copolymers containing hydrophobic drugs have been reported by various research groups [6,7]. However, these nanoparticles had no targeting groups for specific tumor cells. To solve the problem of tumor-specific targeting, some authors have attempted to increase the tissue specificity of the drug carriers by coupling targeting agents, such as monoclonal antibodies, peptide, biotin, and folic acid for delivery of anticancer drug to tumor [8,9].

Hyaluronic acid (HA) is a naturally occurring glycosaminoglycan distributed throughout the extracellular matrix, connective tissues, and organs of all higher animals. The structure of HA consists of repeating disaccharide units of D-glucuronic acid and (1-β-3) *N*-acetyl-D-glucosamine. The molecular weight (MW) typically ranges from 1×10^5 to 5×10^6 daltons [10]. The unique physiochemical and biological properties of HA have highlighted it as a potential targeted macromolecular carrier of drugs to solid tumors. From a biological perspective, the identification of the overexpression of activated HA receptors CD44 and RHAMM on tumor cells that are lacking on their non-tumorigenic counterparts [11-14] and the ability of CD44 to internalize HA [15] reinforced its potential as a targeted drug transport vehicle. This application of HA has been previously recognized, and several preclinical studies have chemically conjugated HA to cytotoxic agents with an end result of highly effective anticancer agents in vitro [16,17].

Our approach in this study used HA as a drug carrier into which poly(lactide-co-glycolide) (PLGA)-linked oligomers of the HA repeat units are attached by diamine PEG as spacer. HA-anchored nanoparticles will have increased exogenous HA concentration in ascites tumor fluid. The high molecular weight of HA is believed to be involved in inhibition of tumor metastasis. DOX-loaded surface-modified nanoparticles are able to deliver DOX within the tumor by receptor-mediated endocytosis and the enhanced permeability and retention effect. This approach will sustain the drug release pattern as well as lead to targeting of the drug.

Material and methods

HA (sodium salt, MW 5740 daltons) was generously supplied by Cadila Health Care (Ahemdabad, India); PLGA (50:50, MW 40–75 kilodaltons, with inherent viscosity 0.59 dL/g), stannous octoate, PEG-2000 (MW 2000 daltons), MPEG 2000 (MW 2000 daltons), and dialysis membrane (MW cutoff 2000) were purchased from Sigma-Aldrich (St. Louis, MO). DL-lactide and glycolide were purchased from Boehringer Ingelheim Pharma GmbH & Co.KG (Ingelheim, Germany). Doxorubicin hydrochloride was supplied as a gift sample from M/s Sun Pharma Advanced Research Center (Vadodara, Gujarat, India). EDAC (1-ethyl-3(dimethylaminopropyl) carbodiimide), Pluronic F-68 was purchased from Himedia Lab (Mumbai, India). *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinamide (NHS), methanol, and toluene were purchased from Spectrochem Pvt. (Mumbai, India). Dimethyl sulfoxide (DMSO) and acetone were procured from Qualigen Fine Chemical (Mumbai, India). All other chemicals used were of analytical reagent grade and were used as received.

Copolymer synthesis and characterization

Diamine PEG was synthesized according to the method reported by Zalipsky et al. [18]. One gram of PLGA dissolved in methylene chloride was activated by 50 mg of DCC and 21 mg of NHS at room temperature (25±2 °C) under a nitrogen atmosphere for 24 hours. The resultant solution was filtered and precipitated by dropping into ice-cold diethyl ether, and the activated PLGA was completely dried under vacuum. The activated PLGA (1 g) dissolved in 8 mL of DMSO was added slowly to 2.1 g of diamine PEG dissolved in 10 mL of DMSO in a dropwise manner with gentle stirring. The reaction was carried out for 6 hours under a nitrogen atmosphere, and the resultant solution was precipitated by addition of ice-cold diethyl ether. The precipitated product, amine-terminated di-block copolymer,

PLGA-PEG-NH_2, was filtered and dried. The free amine groups were determined by using the ninhydrin test. HA-conjugated copolymer was synthesized by coupling the PLGA-PEG-NH_2 di-block copolymer with an activated HA [carboxyl group of HA (1 mmol) EDAC (1.2 mmol) in distilled water]. The reaction was performed at room temperature for 7 hours and then mixed with 50 mL of distilled water and centrifuged at 3000 rpm. After discarding the pellet, the supernatant was dialyzed and dried. The obtained polymer named HA-PEG-PLGA was dried under vacuum overnight. Copolymers were characterized by infrared (IR) spectroscopy using KBr pellet method after adsorption of a smaller amount of copolymer on KBr pellets in a Perkin-Elmer IR spectroscope (Buckinghamshire, UK). Nuclear magnetic resonance (NMR) spectroscopy of the copolymer sample was carried out at 300 MHz, after dissolving first in D_2O and then in $CDCl_3$ as solvent (Bruker DRX 300 MHz, Billerica, MA).

Preparation of DOX-loaded HA-PEG-PLGA nanoparticles

DOX-loaded HA-PEG-PLGA nanoparticles were prepared by the nanoprecipitation method. Briefly, 10.6 mg of DOX HCl in the presence of 6.2 mg of triethylamine was stirred for 24 hours at room temperature under nitrogen. HA-PEG-PLGA copolymer (10 mg) was dissolved in 10 mL acetone. DOX solution was added to the stirred copolymer solution. This organic solution was added dropwise to 20 mL distilled water (or with different amounts of Pluronic F-68) under magnetic stirring. The nanoparticles were formed immediately, and the solvent was removed by overnight evaporation at room temperature. The resulting suspension was centrifuged for 30 minutes at 10,000 rpm. The supernatant was discarded, and the nanoparticles were resuspended in water. Formulations were optimized using process variables such as the amount of copolymer and different concentrations of aqueous Pluronic F-68. The amount of copolymer (10 to 30 mg) was varied while keeping Pluronic F-68 concentration constant (0.50% w/v). Similarly nanoparticles were also prepared using different concentrations of Pluronic F-68 (0 to 0.1% w/v) while keeping the amount of copolymer constant (10 mg).

Preparation of DOX-loaded MPEG-PLGA nanoparticles

The MPEG-PLGA (ratio 5:45) copolymers were synthesized according to the method reported by Beletsi et al., [19] In brief, L-lactide, D-glycolide, and MPEG were polymerized in bulk at 160°C using stannous octoate as the catalyst. Synthesis was confirmed by IR and NMR spectroscopy. DOX-loaded nanoparticles of MPEG-PLGA were prepared as mentioned earlier.

Characterization of the nanoparticles

Morphology

The shape and surface morphology of both HA-PEG-PLGA and MPEG-PLGA nanoparticles were investigated using transmission electron microscopy (TEM). TEM was carried out to determine the surface characteristics of the nanoparticles in aqueous medium using 3-mm Forman (0.5% plastic powder in amyl acetate)-coated copper grid (300 mesh) at 60 kV using negative staining by 2% phosphotungstic acid at various magnifications (Morgagni 268D, Fei, The Netherlands).

Particle size measurements

Both HA-PEG-PLGA and MPEG-PLGA nanoparticles were suspended in distilled water and placed into the cuvette of a laser diffraction particle size analyzer (Malvern-Zetasizer 3000hs, Malvern, UK), and the particle sizes were determined using the software provided by the manufacturer.

Differential scanning calorimetry

The transition temperatures and melting temperatures of polymers (HA-PEG-PLGA and MPEG-PLGA) and DOX-loaded nanoparticles were measured by differential scanning calorimetry (DSC; TA Instrument 2910 MDSC V4.4E, Shin, Osaka, Japan) under a flow of nitrogen at a scanning rate of 10°C per minute. The thermograms were obtained at 0°C to 400°C for blank HA-PEG-PLGA nanoparticles and DOX-loaded HA-PEG-PLGA nanoparticles and at 0°C to 100°C for black MPEG-PLGA nanoparticles and DOX-loaded MPEG-PLGA nanoparticles.

X-ray diffraction (XRD) measurement

X-ray diffractograms of DOX, HA-PEG-PLGA (without DOX), and DOX-loaded HA-PEG-PLGA nanoparticles were obtained with an x-ray diffractometer (Rigaku D/Max-1200; Rigaku Denki Co., Tokyo, Japan) using Ni-filtered CuKa radiation (35 kV, 15 mA).

Drug content

Two milliliters of nanoparticle formulations (HA-PEG-PLGA and MPEG-PLGA) were freeze-dried. Then the dried nanoparticles were redissolved in acetone. The obtained suspension was then centrifuged (Remi, Mumbai, India) at 3000 rpm for 2 minutes. The supernatant was analyzed using spectrophotometer (GBC Cintra 10, GBS Scientific Equipment Pty Ltd., Victoria, Australia) at 478.4 nm.

In vitro *release.* The in vitro release experiment was carried out as reported earlier by Jeong et al. [20]. DOX-loaded HA-PEG-PLGA and MPEG-PLGA nanoparticles (10 mg) were suspended in 2 mL of phosphate-buffered saline (PBS) (pH 7.4) and subsequently put into a dialysis tube (MW cutoff 2000 daltons). The dialysis tube was placed into a 100-mL bottle containing 50 mL PBS, and the medium was stirred at 100 rpm at 37°C. A whole-medium change method was used for prevention of drug saturation in the drug release study. At specific time intervals the whole medium (50 mL) was withdrawn completely and replaced with the same volume of fresh PBS (50 mL). The concentration of released DOX in the PBS was determined by ultraviolet spectrophotometer (GBC Cintra 10) at 478.4 nm.

Hemolytic toxicity

Whole human blood was collected in HiAnticlot blood collection vials (Himedia Labs, Mumbai, India). The red blood cells (RBCs) were separated by centrifugation and resuspended in normal saline solution (10% hematocrit). One milliliter of RBC suspension was incubated separately with distilled water (taken as 100% hemolytic standard); normal saline (taken as blank for spectrophotometric estimation); DOX; HA-PEG-PLGA and MPEG-PLGA nanoparticulate formulations containing equivalent amount of DOX (200 μg/mL) after making up the volume to 10 mL with normal saline. The sample tubes were allowed to stand for 1 hour at 37°C with intermittent shaking. Then the tubes were centrifuged for 15 minutes at 3000 rpm, and the absorbance of supernatant at 540 nm was used to estimate percentage hemolysis against absorbance for supernatant of 100% hemolytic standard (distilled water) diluted similarly.

Hematological study

Healthy male albino rats (Sprague-Dawley strain) of uniform body weight (100 ± 20 g) with no prior drug treatment were used for all the present in vivo studies. The rats were maintained on standard diet and water. The protocol was duly approved by the Institutional Animal Ethics Committee of Dr. H.S. Gour University, Sagar (M.P.) (registration no. 379/01/ab/CPCSEA, India).

The animals were divided into six groups having six rats in each group. Plain DOX solution, DOX-loaded HA-PEG-PLGA nanoparticles, HA-PEG-PLGA nanoparticles (without DOX), DOX-loaded MPEG-PLGA nanoparticles, and MPEG-PLGA (without DOX) nanoparticles, each containing an equivalent amount of DOX (240 μg/kg) were administered intravenously to the first, second, third, fourth, and fifth group every day. The sixth group was kept as control, which was maintained on the same regular diet for 7 days. For hematological evaluation, RBC and platelet counts were measured on day 7. In addition, plasma concentrations of blood urea nitrogen and creatinine were also measured.

Radiolabeling on nanoparticulate formulation

^{99m}Tc-labeled nanoparticulate formulation was prepared by simple reducing method using $SnCl_2 \cdot H_2O$ as reducing agent. Radiolabeled compound was prepared by dissolving $SnCl_2 \cdot H_2O$ 1 mg/mL. To 50 μL of nanoparticulate formulation an equal amount of stannous chloride solution (made in 10% acetic acid) was added. The pH of the solution was measured and adjusted to 7.0, if required. The solution was then passed through a 0.22-μm Millipore filter (Billerica, MA). After 4 mCi of activity (^{99m}Tc) was added dropwise, the mixture was incubated at room temperature for 10 minutes. Radiolabeling efficiency on nanoparticles was determined using ITLC-SG (instant thin layer chromatography using silica gel coated fiber sheet from Gelman Science Inc., Anne Arbor, MI) strips with saline and acetone as the mobile phase at 15 and 60 minutes after filtering. The radiolabeling efficiency of label entrapment in the nanoparticles was always higher than 90%.

Biodistribution study of ^{99m}Tc-labeled DOX-loaded HA-PEG-PLGA nanoparticulate formulation

Balb/C mice (aged 2–3 months) weighing between 25 and 30 g were selected for the study. Ehrlich ascites tumor (EAT) cells were grown in the ascites fluid of mice by injecting 2.5×10^7 cells intraperitoneally. The cells were harvested on day 7 after administration. These ascites cells were subcutanously inoculated in soft tissue to produce solid tumor.

The tumor-bearing mice (weight 30 ± 5 g) were used to study the biodistribution of DOX and its nanoparticulates formulation. The mice were divided into six groups of six animals each. The mice of the first group were injected with 300 μg of HA-PEG-PLGA copolymer in PBS (pH 7.4) into their tail vein; the second group of mice was injected into the tail vein with ^{99m}Tc-labeled DOX-loaded HA-PEG-PLGA nanoparticles, and the third group was injected with plain ^{99m}Tc-labeled DOX (240 μg/kg). The fourth group of mice was injected in the tail vein with plain ^{99m}Tc-labeled DOX-loaded MPEG-PLGA (240 μg/kg). The fifth group of mice was injected in the tail vein with ^{99m}Tc-labeled MPEG-PLGA (300 μg/kg). The sixth group of mice was kept as control. After 1, 2, and 4 hours the mice were killed by cervical dislocation, and their tissues (e.g., tumor, liver, spleen, lungs, kidney, intestines, heart, stomach, and muscle) were excised. After washing these quickly with cold water to remove surface blood, radioactivity was counted. Blood samples (1 mL) were obtained in duplicate by cardiac puncture in preweighed heparinized tubes. The radioactivity remaining in the tail was also measured and taken into consideration in the calculation of total radioactivity dose administered to the mice.

Tumor growth inhibition study

Tumor growth inhibition effect of the DOX-loaded HA-PEG-PLGA nanoparticulate formulation on the growth of EATs was also studied. Tumor-bearing mice (tumor size 50–100 mm^3) were allocated to three groups of six animals in each. The first group was injected in the tail vein with DOX-loaded HA-PEG-PLGA nanoparticles; mice in the second group were injected in the tail vein with plain DOX. The third group was kept as control. DOX and DOX-loaded HA-PEG-PLGA nanoparticulate formulations were injected (240 μg/ml) daily for 15 days. Tumor volume was measured daily using calipers. The tumor volume was calculated using the following equation:

$$\text{Tumor volume } (V) = \text{length} \times \text{width} \times \text{width}/2$$

Statistical analysis was performed using the paired Student's *t*-test.

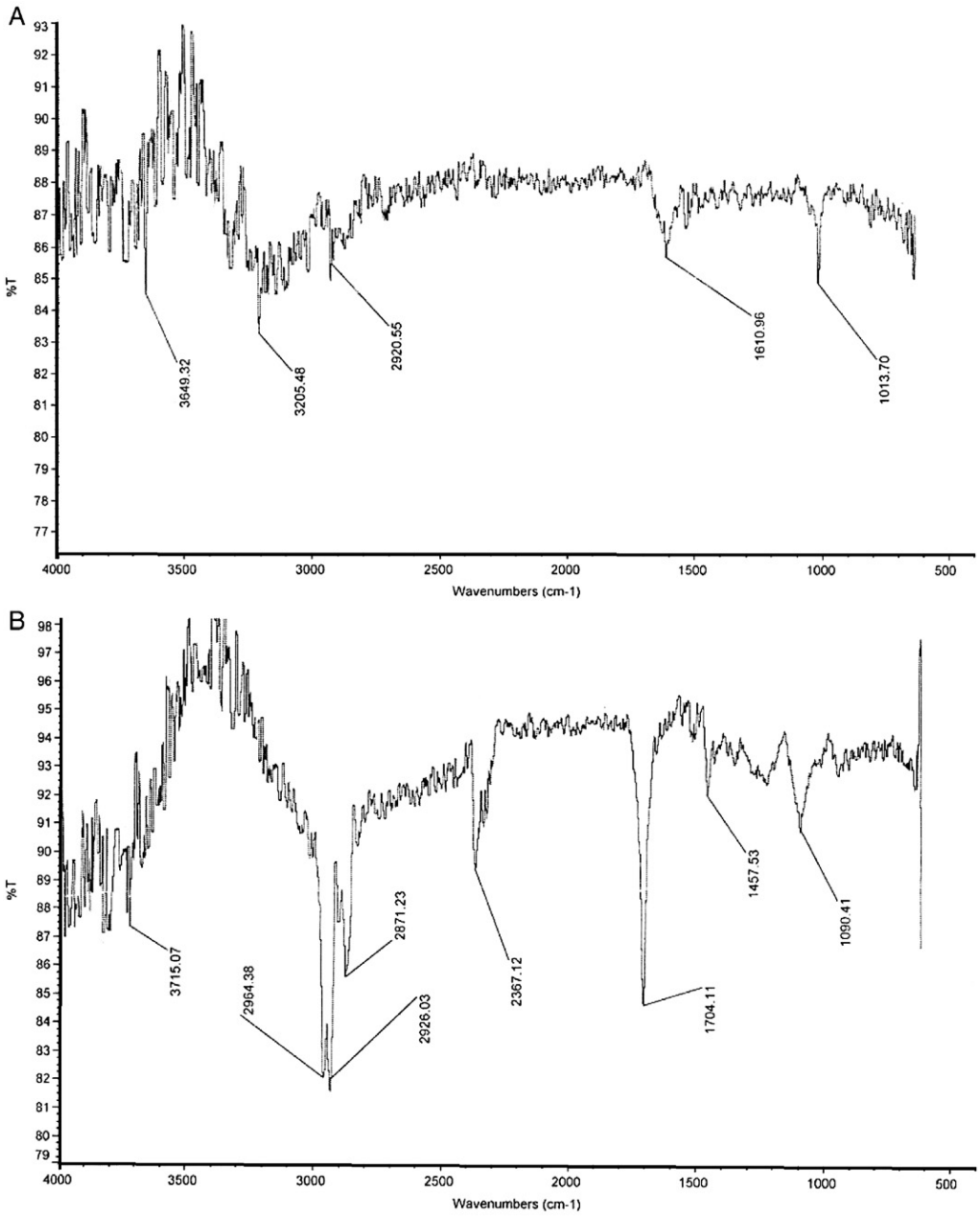

Fig 1. IR spectra of **A**, HA-PEG-PLGA and **B**, MPEG-PLGA.

Results and discussion

Copolymer synthesis and characterization

IR and ^{1}H-NMR spectra of synthesized HA-PEG-PLGA copolymer are shown in Figures 1, *A* and 2, *A* and *B*. Figure 1, *A* confirms the synthesis of copolymer. A typical absorption band of amide (C = O stretch absorption at 1610.96 per cm and N–H bending at 3405.48 per cm), aliphatic C–H stretch at 2920.55 and O–H stretch of alcohol at 3649.32 per cm of HA may be used to confirm the HA-PEG-PLGA conjugate. Synthesis of HA-PEG-PLGA was further confirmed by ^{1}H-NMR of the synthesized copolymer. In D_2O the characteristic peak of the methyl protons of the HA was shown at about 1.4 ppm, whereas a protons peak due to overlap of glucose unit peak of HA and ethylene oxide peak of PEG ethylene oxide of PEG segment are shown at 3.6 ppm. An apparent *N*-acetylate proton peak ($NCOCH_3$) in HA was detected at 2.5 ppm (Figure 2, *A*). In ^{1}H-NMR in $CDCl_3$, the hydrogen of the methine group of the lactic acid unit of the PLGA copolymer resonated at 5.2 ppm, whereas those of the methylene group of the glycolic acid unit appeared at 4.8 ppm (Figure 2, *B*). In this case the MPEG-PLGA copolymer is characteristic of both PEG and PLGA. The

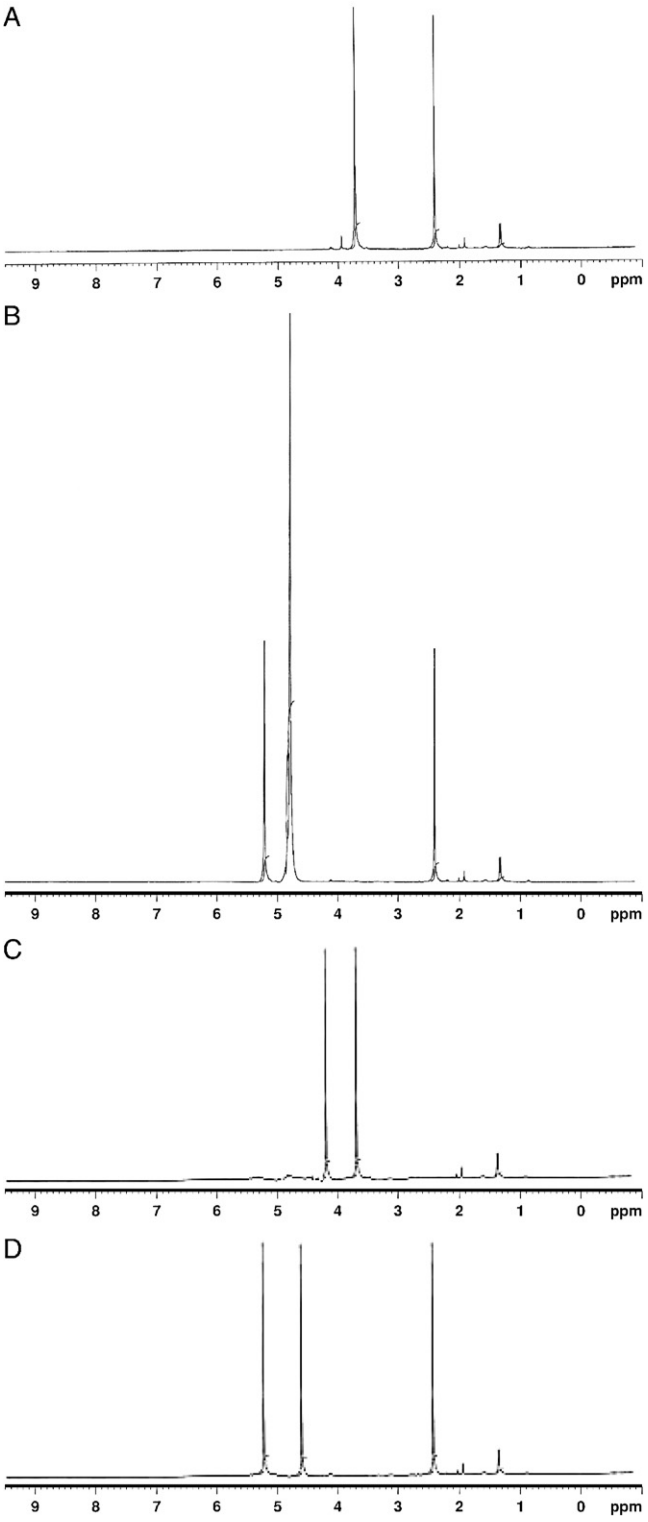

Fig 2. NMR spectra of **A**, **B**, HA-PEG-PLGA and **C**, **B**, MPEG-PLGA.

absorption band at 1704.11 per cm (Figure 1, *B*) is attributed to the C = O stretching (ester C = O stretching) vibrations of the ester carbonyl group. The absorption band at 1090.41 per cm is attributed to O–C stretching. The entire C–H stretching bonds are centered at 2871.23 and 2964.38 per cm. It is characteristic to see the weak absorption band at 3715.07 per cm of alcohol, indicating that free hydroxyl groups of MPEG had reacted with the carboxyl group of lactide/glycolide. All these signals indicate that the MPEG-PLGA block copolymer may be formed. Synthesis of MPEG-PLGA copolymer was also confirmed by ^{1}H-NMR. In D_2O the characteristic peak of PEG was shown at 3.6 to 4.0 ppm. In $CDCl_3$, the hydrogen of the methine group of the lactic acid unit of the PLGA copolymer resonated at 5.2 ppm, whereas those of the methylene group of the glycolic acid unit appeared at 4.8 ppm (Figure 2, *C* and *D*).

Table 1
Process parameter of HA-anchored PLGA nanoparticles

	HA-anchored PLGA nanoparticles		
Serial no.	Fabrication variables	Mean diameter (nm)	Percentage drug entrapment
1. Polymer amount (mg)	10	102 ± 5	94.36
	20	132 ± 5	92.28
	30	176 ± 5	88.74
2. Surfactant concentration (w/v)	0.00	186 ± 6	87.66
	0.050	145 ± 5	93.28
	0.100	107 ± 7	96.74
3. Solvent volume (mL)	10	142 ± 2	92.66
	20	125 ± 5	94.28
	30	93 ± 6	96.92

Table 2
Process parameter of MPEG-PLGA nanoparticles

	MPEG-PLGA nanoparticles		
Serial no.	Fabrication variables	Mean diameter (nm)	Percentage drug entrapment
1. Polymer amount (mg)	10	104 ± 5	95.56
	20	123 ± 3	90.48
	30	132 ± 3	87.74
2. Surfactant concentration (w/v)	0.00	186 ± 2	85.25
	0.050	135 ± 3	90.24
	0.100	117 ± 5	97.74
3. Solvent volume (mL)	10	125 ± 2	90.66
	20	115 ± 4	93.24
	30	98 ± 7	96.86

Preparation of nanoparticles

Tables 1 and 2 show the particle size of the DOX-loaded HA-PEG-PLGA and MPEG-PLGA nanoparticles. It is clear from these tables that the processing variables influenced the size, size distribution, and drug-loading capacity of nanoparticles. Hence the method was optimized to produce nanoparticles of small size and narrow size distribution with high entrapment efficiency. Increasing the amount of HA-PEG-PLGA (copolymer) from 10 to 30 mg caused the

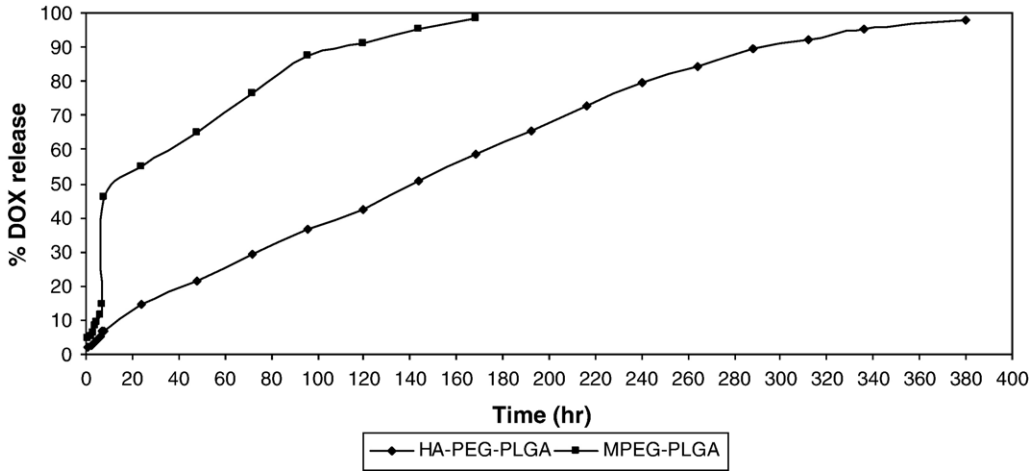

Fig 3. In vitro DOX release studies of HA-PEG-PLGA and MPEG-PLGA.

nanoparticles' size to shift toward a higher size range (from 102 to 176 nm), and entrapment efficacy was also decreased from 94.36% to 88.74%. Higher concentration of copolymer produced a more viscous solution that caused a reduction in the rate of diffusion of copolymer solution in the water phase. It is well accepted that the size of nanoparticles is directly dependent on the rate of diffusion of organic solvent to the outer aqueous environment. The faster the diffusion rate is, the smaller the particles would result [21,22].

The size of the nanoparticles decreased gradually with increasing concentration of surfactant (Pluronic F-68). The size of nanoparticles varied from 186 to 107 nm when the concentration of Pluronic F-68 increased from 0 to 0.1%, but entrapment efficacy was increased from 87.66% to 96.74%. The nanoparticles were spontaneously formed by the interfacial turbulence resulting from the rapid diffusion of water-miscible solvent to water. The energy released in this diffusion process helps in the formation of nanoparticles. The surfactant is actually not involved in the formation of nanoparticles. It is added to keep good steric stability of the formed particles [21,23].

The results of drug-loading efficiency are summarized in Tables 1 and 2. The drug-loading efficiency was higher than 87.66%, and many of the nanoparticulate formulations showed more than 90% of loading efficiency. In case of MPEG-PLGA nanoparticles the entrapment efficiency was higher than 85.25% and also followed a similar pattern (Table 2).

Morphology

The shape of nanoparticles was visualized by TEM, and photomicrographs were taken in dry condition. The nanoparticles were found to be spherical, and the average size is around 100 to 200 nm (photograph not shown).

In vitro DOX release

Figure 3 shows that HA-PEG-PLGA nanoparticles were able to sustain the DOX release over 15 days. On other hand, MPEG-PLGA nanoparticles were able to release DOX for as long as 12 days and longer. This may be attributed to the hydrophobic and hydrophilic nature of HA-PEG-PLGA copolymer, which increased the hydrophilic chain of nanoparticles and hence increased longer circulation time. The more hydrophilic drug tends to be more partitioned into the hydrophilic domain. On the other hand, the more hydrophobic drug tends to be more partitioned into the hydrophobic domain [24].

Differential scanning calorimetry (DSC)

The glass transition temperature (T_g) of polymers has an important effect on the drug release. Both HA-PEG-PLGA nanoparticles and MPEG-PLGA nanoparticles followed a similar pattern. The DSC thermograms for DOX, HA-PEG-PLGA nanoparticles, and DOX-loaded HA-PEG-PLGA nanoparticles are shown in Figure 4. For HA-PEG-PLGA nanoparticles, the peaks that occurred at temperatures above 325.5°C represent the decomposition of the material tested. HA showed the presence of an endothermic peak, with a broad endothermic peak at 80°C for HA. Diamine PEG showed an endothermic peak, which is located at its melting point (T_g) at 20°C. In addition, significant sharp exothermic peaks were observed for HA, indicating the crystalline structure of the HA. The increase of the T_g peak indicated that the HA nanoparticle is crystalline (Figure 4, *A*). When comparing the profiles of DOX-loaded HA-anchored nanoparticles with HA-anchored nanoparticles, a sharp peak of DOX at 220°C and other peaks are seen to be similar to blank HA-PEG-PLGA nanoparticles that showed DOX present in crystalline state (Figure 4, *B*), and plain DOX showed an exothermic peak between 175° to 202°C, as already reported [25]. Similar results were found in the case of MPEG-PLGA nanoparticles (with or without DOX) (Figure 4, *C* and *D*). These results correlate well with similar studies [25,26].

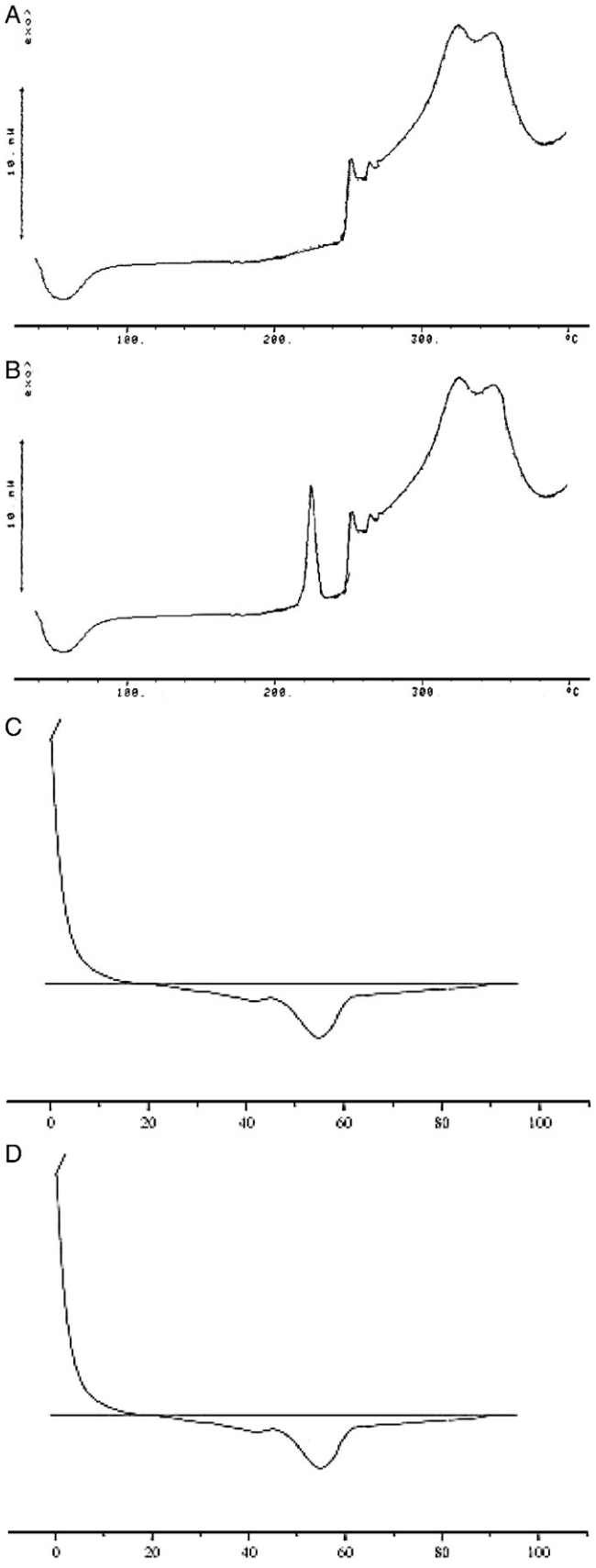

Fig 4. DSC thermograms of **A**, HA-PEG-PLGA, **B**, DOX-loaded HA-PEG-PLGA nanoparticles, **C**, MPEG-PLGA, and **D**, DOX-loaded MPEG-PLGA nanoparticles.

X-ray diffraction (XRD) measurement

Figure 5, *A–C* shows the XRD patterns of HA-PEG-PLGA copolymers, DOX-loaded HA-PEG-PLGA nanoparticles, and DOX. As shown in Figure 5, *A* and *B*, the characteristic crystalline peak of PEG was reduced by copolymerization with L-lactide and D-glycolic acid, whereas the characteristic peak of PLGA was increased (Figure 5, *D* and *E*). The HA-PEG-PLGA copolymer could control the degradation rate and achieved better physicochemical properties and processibility. The number of peaks was increased that showed the increased crystalline nature of copolymer. In particular, it is expected that block copolymers composed of PLGA, PEG, and HA can be easily prepared as nanoparticulate carriers with core-corona type structures. XRD patterns showed typical sharp peaks in drug crystal, whereas empty nanoparticles showed relatively fewer peaks. It was thought that drug crystallites showed sharp specific crystal peaks when existing as drug crystals. These results are well correlated with a similar study [26].

Drug content

The drug content was described earlier, and results are summarized in Tables 1 and 2. The drug content was found to be 87.66% to 96.74% (w/w). Drug content was decreased by increasing the amount of HA-PEG-PLGA copolymer but increased by increasing surfactant (Pluronic F-68) concentration. Similarly, increasing solvent volume increased the drug content of nanoparticles. MPEG-PLGA nanoparticles followed a similar pattern.

Hemolytic toxicity

When we tested the hemolytic activities of free DOX, DOX-loaded HA-PEG-PLGA, and MPEG-PLGA nanoparticles, neither nanoparticulate formulation with HA-PEG-PLGA and MPEG-PLGA copolymers showed any detectable hemolytic activity on RBCs. DOX concentrations used in this study were 100 and 200 μg/mL. In the case of both nanoparticulate formulations, the DOX concentration was calculated based on their drug content (Tables 1 and 2). Both nanoparticulate formulations prepared with HA-PEG-PLGA and MPEG-PLGA copolymers showed neglible hemolytic toxicity on RBCs in the whole experimental DOX concentration range (100 and 200 μg/mL). DOX-loaded HA-PEG-PLGA and MPEG-PLGA nanoparticles were found to be apparently more hemocompatible for drug delivery applications. The suppression of hemolytic toxicity correlated well with other similar studies of PCL-PEG micelles [6].

Hematological study

To prove the safety of nanoparticulates as drug carriers, subacute toxicity for nanoparticulates themselves was

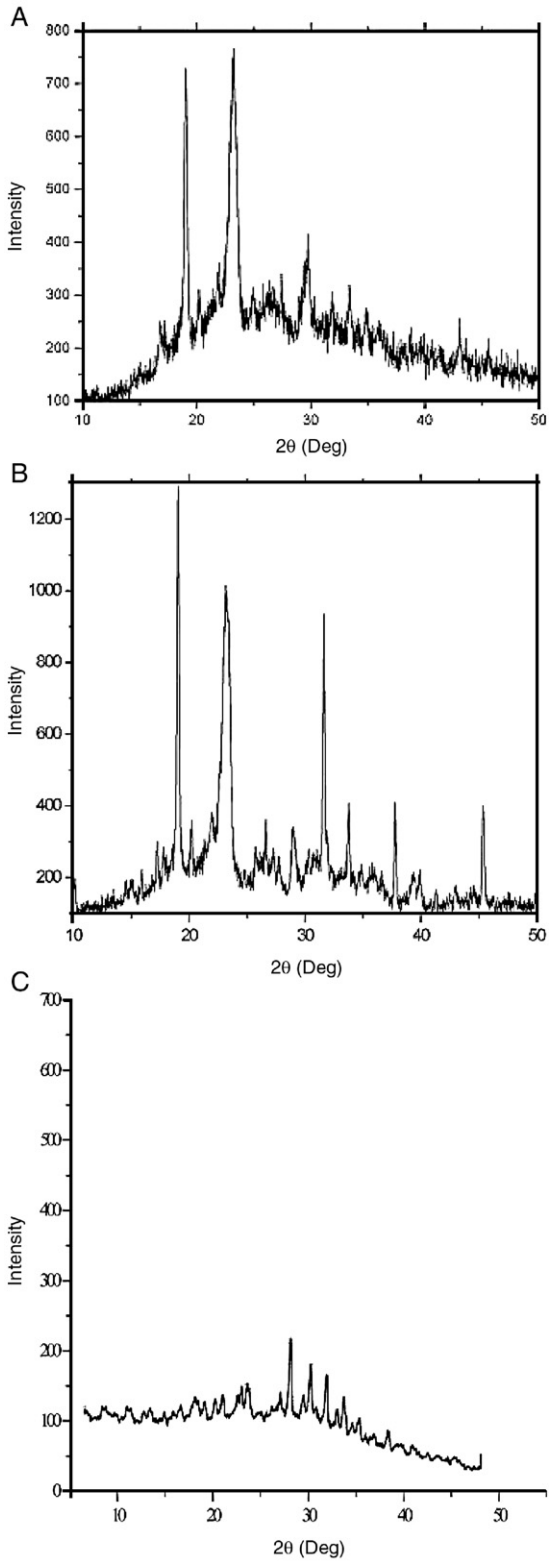

Fig 5. X-ray diffractograms of **A**, HA-PEG-PLGA nanoparticles (without DOX), **B**, DOX-loaded HA-PEG-PLGA nanoparticles, **C**, DOX, **D**, diamine PEG, and **E**, PLGA.

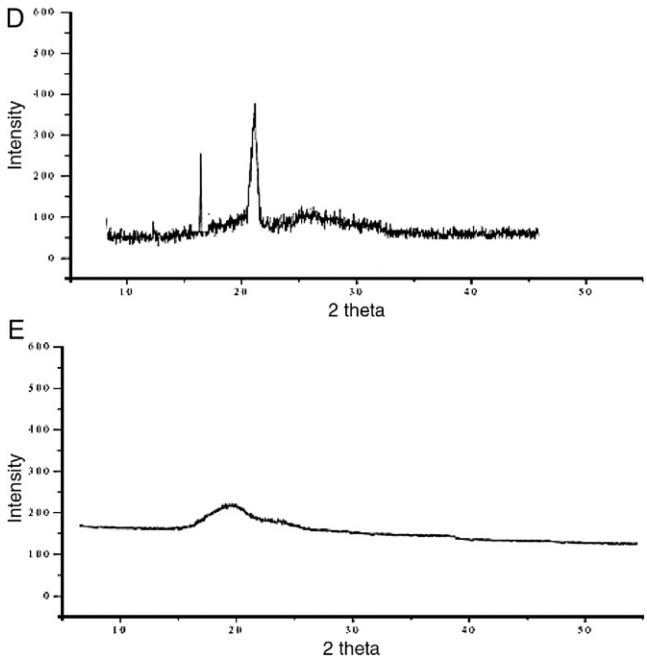

Fig 5 (*continued*).

evaluated in vivo after repeated injection through the lateral tail vein of mice at various dosages for 7 days. In mice that received the treatment of nanoparticulate formulations at a dose of 240 μg/kg, the RBCs and platelet counts were not changed compared to the control group. Creatinine and blood urea nitrogen were normal in all groups, indicating that the liver and renal functions were normal. This means that the nanoparticulate formulations do not cause unexpected side effects and that they are safe drug carriers (data not shown). However, Park et al. [27] have reported that DOX itself at a dosage of 2 mg/kg daily for 3 days and 5 mg/kg daily for 6 days caused a significant loss of body weight as compared with the nanoparticulate formulation, respectively. They have also reported in vivo subacute toxicity of DOX-loaded nanoparticles prepared by heparin–deoxycholic acid chemical conjugate. From this study it may be concluded that the HA-anchored PLGA nanoparticulate formulation is safer for intravenous delivery of DOX.

Biodistribution study of ^{99m}Tc-labeled DOX-loaded HA-PEG-PLGA nanoparticulate formulation

To evaluate the potential significance of the nanoparticles' uptake by various tissues with regard to a cytotoxic drug, the biodistribution study was performed using DOX with ^{99m}Tc either as free complex or encapsulated in nanoparticles. These experiments were performed in EAT-bearing mice models. The percentage injected dose per gram of tissue in different organs at different time intervals for free drug and its nanoparticles is shown in Figure 6, *A–C*. As expected, HA-PEG-PLGA nanoparticles showed greater activity in tumor in comparison with plain DOX. It is clear from the tumor concentrations of DOX that HA-PEG-PLGA nanoparticulate formulations could deliver a significantly

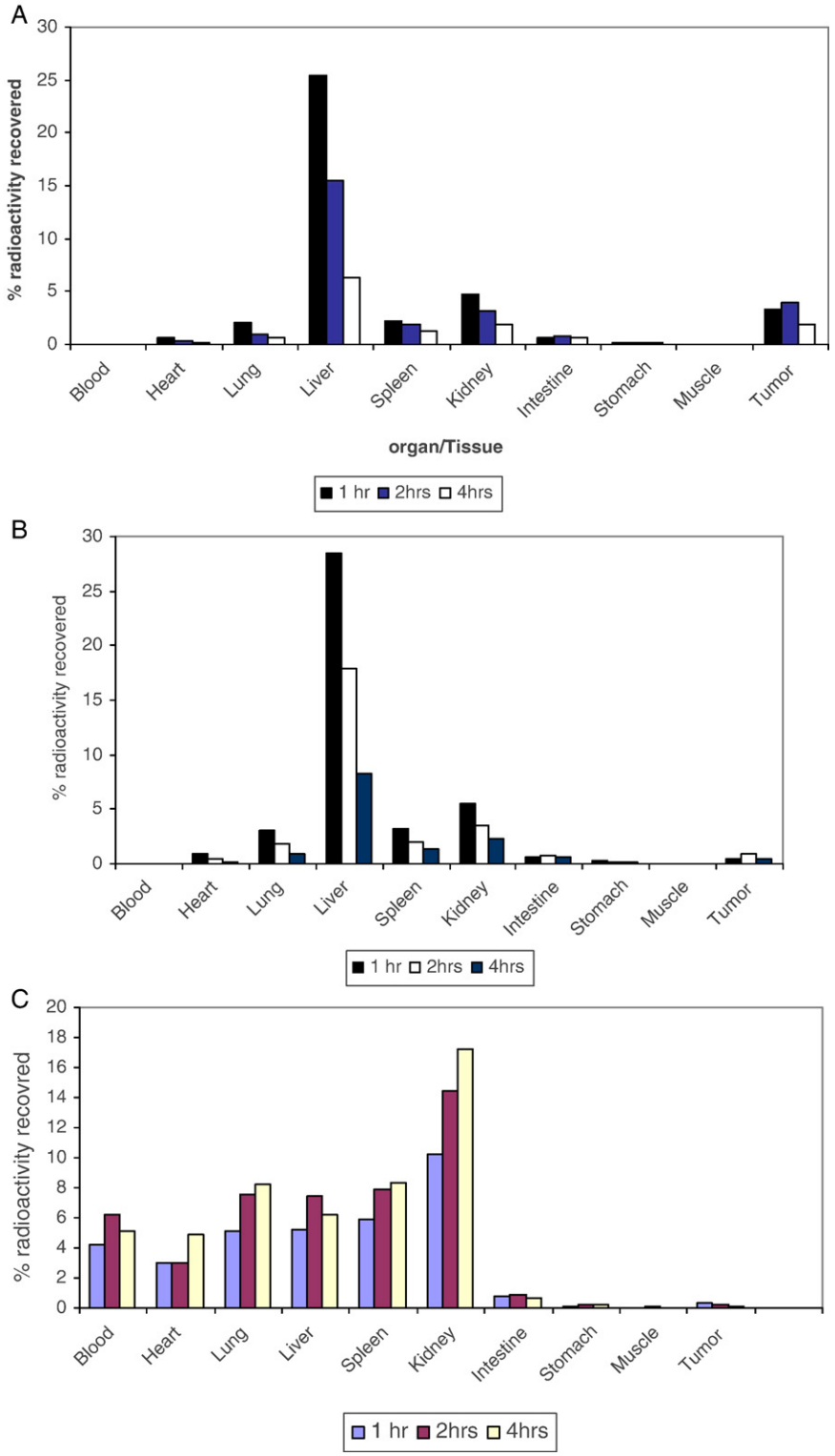

Fig 6. Biodistribution of (A) DOX-loaded HA-PEG-PLGA, (B) DOX-loaded MPEG-PLGA nanoparticles in EAT-bearing mice, and (C) DOX.

higher concentration of DOX into the tumor than DOX-loaded MPEG-PLGA and plain DOX solution. At 1 hour after administration, for instance, there was a fourfold higher concentration of DOX in the tumor when delivered in HA-PEG-PLGA nanoparticles and a twofold higher concentration with MPEG-PLGA nanoparticles as compared with the aqueous solution (Figure 7). Results were even more pronounced at 2 hours after administration. This may be

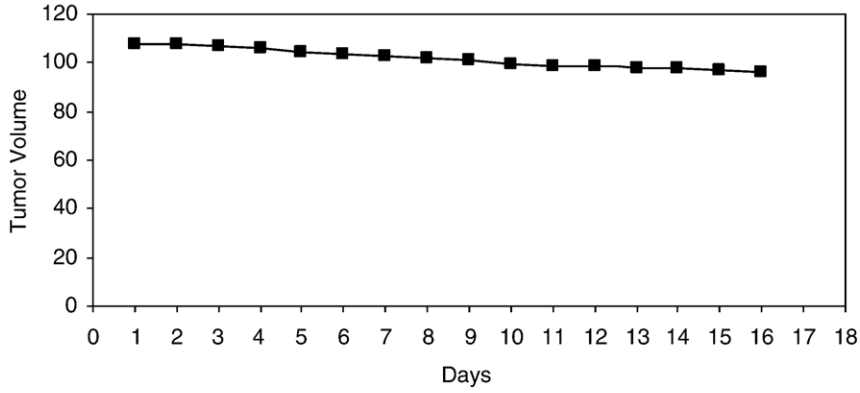

Fig 7. Tumor inhibition study of DOX-loaded HA-PEG-PLGA nanoparticles in EAT-bearing mice.

attributed to the higher efficiency of nanoparticles to accumulate within the tumor mass predominantly by virtue of the enhanced permeability and retention mechanism and competing with endogenous HA and exogenous HA [28-30].

Tumor growth inhibition study

The in vivo activity for tumor growth inhibition was performed in an EAT tumor model with established tumors using a single dose of the formulations. Starting tumor size of treated groups was not statistically significantly different from the control group. Although the free-DOX dose was initially efficient in suppressing further tumor growth inhibition activity did not last long. The response of the nanoparticulate formulations in the EAT tumor model was not significantly different, and treated groups could not be distinguished from the control group, except during the first 24 hours after injection when HA-PEG-PLGA or MPEG-PLGA were found to be significantly more active than DOX in solution, with a reduction in size between 1 day and 2 days after injection being significant for all the nanoparticulate formulation groups. In all nanoparticle groups as well as the control group, all animals appeared lively throughout the study, and no considerable weight loss was detected. There were no signs of decreased activity, which could indicate general toxicity. Thus, nanoparticulate formulations may be considered to be safe at the dosing schedule used. DOX-loaded HA-PEG-PLGA nanoparticulates have a size of about 96.5 mm^3. Similar findings have been reported by Mo and Lim [31], who used wheat germ agglutinin–anchored PLGA nanoparticles for an SCID mice model engrafted with the A549 tumor nodule, and they obtained no substantial loss in body weight. Indeed, the results demonstrated that HA facilitates the recognition of nanoparticles by EAT; after cell surface binding the nanoparticles may be internalized into the targeted cells by receptor-mediated endocytosis.

Anghileri [32] found that the acid mucopolysaccharide present in the ascitic fluid supernatant is HA. HA seems to be of extracellular origin, and it is bound to proteins of the cell membrane. The ascites cells show a very active production of sulfated mucopolysaccharides particularly at the mitochondrion and cell membrane level. HA-PEG-PLGA nanoparticles increased the HA level, suggesting that exogenous oligomeric HA inhibits tumor progression, most likely by competing with endogenous polymeric HA [29,30].

Conclusion

HA-PEG-PLGA nanoparticles were a suitable sustained-release carrier for DOX as compared with MPEG-PLGA nanoparticles. HA-PEG-PLGA nanoparticles were found to be hemocompatible and nontoxic. Specific targeting to tumor tissue is possible using HA-PEG-PLGA nanoparticles. HA-PEG-PLGA nanoparticles seemed to be equally effective in targeting the EAT tumor model. In this model the HA-PEG-PLGA nanoparticles showed long tumor retention times with rapid clearance from normal tissues.

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