Pharmaceutical Research, Vol. 26, No. 3, March 2009 (© 2008)
DOI: 10.1007/s11095-008-9769-y

Research Paper

Synthesis and Evaluation of Pegylated Dendrimeric Nanocarrier for Pulmonary Delivery of Low Molecular Weight Heparin

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Received May 30, 2008; accepted October 24, 2008; published online November 25, 2008

Purpose. This study tests the hypothesis that pegylated dendrimeric micelles prolong the half-life of low molecular weight heparin (LMWH) and increase the drug's pulmonary absorption, thereby efficacious in preventing deep vein thrombosis (DVT) in a rodent model.
Materials and Methods. Pegylated PAMAM dendrimer was synthesized by conjugating G3 PAMAM dendrimer with methyl ester of polyethylene glycol 2000 (PEG-2000). Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR) spectra and thin layer chromatography (TLC) were used to evaluate the identity and purity of pegylated dendrimer. The particle size distributions of the formulations were measured by using a Nicomp Zeta meter, and drug entrapment efficiency was studied by azure A assay. The efficacy of pegylated dendrimers in enhancing pulmonary absorption, prolonging drug half-life, and preventing DVT was studied in a rodent model.
Results. FTIR, NMR and TLC data confirmed that PAMAM dendrimer was conjugated to PEG-2000. The entrapment efficiency of LMWH in PEG–dendrimer micelles was about 40%. Upon encapsulation of LMWH, the particle size of PEG–dendrimer micelles increased from 11.7 to 17.1 nm. LMWH entrapped in PEG–dendrimer produced a significant increase in pulmonary absorption and the relative bioavailability of the formulation was 60.6% compared to subcutaneous LMWH. The half-life of the PEG–dendrimer-based formulation was 11.9 h, which is 2.4-fold greater than the half-life of LMWH in a saline control formulation. When the formulation was administered at 48-h intervals, the efficacy of LMWH encapsulated in pegylated dendrimers in reducing thrombus weight in a rodent model was very similar to that of subcutaneous LMWH administered at 24-h intervals.
Conclusions. Pegylated PAMAM dendrimer could potentially be used as a carrier for pulmonary delivery of LMWH for the long-term management of DVT.

KEY WORDS: jugular vein thrombosis; low molecular weight heparin; pegylated PAMAM dendrimer; pulmonary absorption.

INTRODUCTION

Dendrimers are a new family of man-made, highly branched and globular shaped macromolecules with tremendous promise in drug delivery. The potential use of dendrimers in drug delivery and their unique chemical and physical properties have recently been reviewed (1–8). To serve as a drug carrier, dendrimers interact with a drug molecule by a number of mechanisms: (1) Drugs can be encapsulated within dendrimeric cavities, where they may be retained by physical entrapment, electrostatic interactions, hydrophobic or hydrogen bonding; (2) Drugs can also be bound to the surface of the dendrimer by electrostatic or covalent interactions. Such interactions between dendrimers and drugs can be exploited to enhance drug solubility and bioavailability, provide controlled release, and offer opportunities for targeted drug delivery (9). However, safety and drug loading capacity are the major determinants governing whether a polymer can be used a drug carrier. Although low-generation dendrimers (generation 4 or lower) have desirable biological properties, such as a low toxicity and untoward immunogenicity *in vivo*, they cannot entrap the same amount of guest molecules as high-generation dendrimers because of their relatively small inner cavities and limited reaction surfaces (9,10).

PEGs have excellent solubility in aqueous and many organic solutions. Being nontoxic and nonimmunogenic, PEGs have favorable pharmacokinetics and tissue distribution. Because of these favorable features, conjugation of PEGs with dendrimers is likely to further improve their biocompatibility. In fact, attachment of PEGs to dendrimers may lead to an increase in the inner cavity space of dendrimers, thereby increasing their drug-loading capacity to facilitate delivery of large molecular weight compounds (9). Furthermore, pegylation can also increase the circulation time of the drug in the blood by masking it from recognition by the reticuloendothelial system (11).

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Low molecular weight heparins (LMWHs) are negatively charged oligosaccharides used in the treatment of deep vein thrombosis (DVT) and pulmonary embolism. However, the use of LMWHs on an outpatient basis has been limited because of the requirement of daily subcutaneous injections. Consequently, attempts have been made to deliver LMWHs via noninvasive routes, including the nasal and pulmonary routes (9,12–17). Previously, we showed that, similar to DNA–dendrimer complexation, a full-generation cationic PAMAM dendrimer interacts with electronegative functional groups of LMWHs and increases the pulmonary absorption of the drug (17). However, it is not known if pegylated dendrimers can be used to increase pulmonary absorption and circulation time of the drug. Moreover, there are no data as to the feasibility of encapsulating LMWH into pegylated dendrimers. Therefore, this study was designed to test (1) whether pegylated dendrimers, when used as a nanocarrier, can increase the circulation time of LMWH, and (2) whether a long-circulating pegylated dendrimer-based formulation of LMWH can enhance pulmonary absorption and prevent DVT in a rodent model.

MATERIALS AND METHODS

Materials

LMWH (average molecular weight and anti-factor Xa activity are 4,494 Da and 60 U/mg, respectively) was purchased from Celsus Laboratories (Cincinnati, OH). A 20% solution of generation 3 (G3) PAMAM dendrimer in methanol, azure A, 4-nitrophenyl chloroformate chloride, and polyethylene glycol 2000 monomethyl ester (mPEG) were purchased from Sigma-Aldrich (St. Louis, MO). Deuterium oxide (D_2O, 99.9%) was obtained from Cambridge Isotope Laboratories (Andover, MA).

Synthesis of mPEG–Dendrimer Micelles

Pegylation of G3 PAMAM dendrimers was performed according to the procedure shown in Fig. 1. The product was purified according to a previously published method after slight modification (18–20). Briefly, 0.8 g mPEG (MW=2,000) (0.4 mmol) was dissolved in 40 ml tetrahydrofuran (THF) and added to 201.5 mg of 4-nitrophenyl chloroformate (1.0 mmol) and 56 μl of triethylamine (0.4 mmol) at 4°C. The resulting mixture was then stirred for 24 h at room temperature. The salt was filtered off and the filtrate was concentrated to 10 ml and added dropwise to diethyl ether to precipitate as activated mPEG *p*-nitrophenyl chloroformate. The mPEG *p*-nitrophenyl chloroformate precipitate was further dissolved in THF and purified by reprecipitation from THF into diethyl ether. Next, 27.6 mg of G3.0 PAMAM dendrimers solution (4 μmol) was dissolved in dimethyl sulfoxide (10 ml), and 52 mg (256 μmol) mPEG *p*-nitrophenyl carbonate was added to the solution at 4°C and then the solution was stirred for 72 h at room temperature. The reaction mixture was evaporated to dryness using a rotary evaporator. The obtained product was dissolved in distilled water and the low molecular weight reagents were removed via ultrafiltration using a Cellu Spe T3 membrane (Membrane Filtration Products, Inc. Seguin, TX, MWCO: 12,000–14,000). The dialyzed product was then lyophilized and used in further characterization.

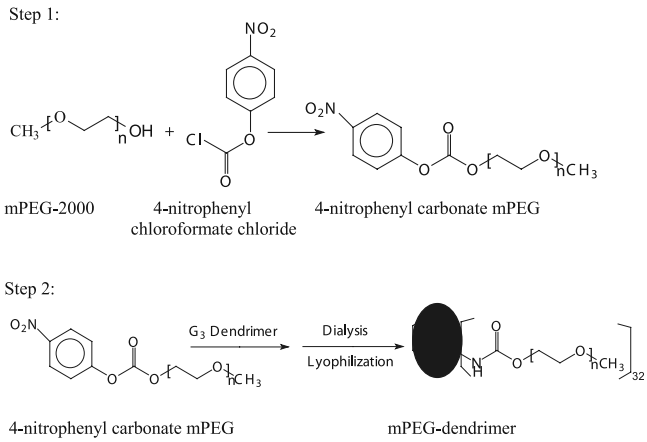

Fig. 1. Schematic diagram showing the synthesis of mPEG–dendrimer.

Thin Layer Chromatography (TLC)

TLC aluminum plates (Silica gel 60 F254, Merck kGaA, Darmstadt, Germany) were used to monitor the progress of the reaction and evaluate the purity of the products. Reactants and products were dissolved in chloroform or water, respectively. The reaction media or solutions of purified products and reactants (0.1 g/ml) were spotted onto a start line on one side of the TLC plates. In TLC chambers, chloroform/methanol (2:1, *v*/*v*) and methanol/water (2:1, *v*/*v*) were used to develop the plates in the first and second steps, respectively. After evaporation of developing solvents, chromatographic spots of samples were visualized by exposing them to iodine vapor.

FTIR and ^{1}H NMR Studies

Attenuated total reflectance Fourier transform infrared (FTIR) spectra of reactants, synthetic pegylated PAMAM dendrimers were recorded on a Nicolet Nexus 470 spectrometer (Thermo Nicolet Corp., Madison, WI) using the Smart Miracle ATR accessory. Samples (10 mg or 50 μl) were applied to the center of the sample holding device and scanned between 4,000 and 700 cm^{-1} at a resolution of 1 cm^{-1}. ^{1}H NMR spectra were recorded on a Varian Mercury Plus 300 MHz spectrometer (Varian, Inc., Fort Collins, CO). The internal standard was tetramethylsilane. Ten milligrams of each sample were dissolved in the deuterated solvent, filtered, and degassed before the measurement. The chemical shift of D_2O was at 4.8 ppm.

Loading of LMWH in mPEG–Dendrimer Micelles

mPEG–dendrimer plus LMWH formulations were first prepared by mixing an aliquot of mPEG–dendrimer solution and LMWH. Before mixing, the drug and polymer solutions were diluted in saline. The resulting solution was then incubated for 30 min at room temperature for complexation to occur. The loading of LMWH in the mPEG–dendrimer micelles was determined by a colorimetric assay using azure A blue dye as described previously (17). For this test, a fixed

amount of azure A dye solution was mixed with the following test samples: 0.125%, 0.25%, 0.5%, 1%, and 2% (*w*/*v*) solutions of mPEG–dendrimers–LMWH formulations containing 5 mg/ml of LMWH. The samples were then measured by a microtiter-plate reader (TECAN U.S. Inc, Research Triangle Park, NC) at 595 nm.

Particle Size Determination of mPEG–Dendrimer Micelles Containing LMWH

The optimized LMWH formulation in the mPEG–dendrimer was characterized by measuring particle size. For particle size determination, plain pegylated PAMAM dendrimer (1%, *w*/*v*) and its formulation with LMWH (5 mg/ml) were prepared. The prepared sample solutions (~300 μl) were dispensed in disposable tubes and particle size measurements were performed in triplicate using a NICOMP™ 380 ZLS, PSS-Nicomp particle sizing system (Santa Barbara, CA).

Pulmonary Absorption Studies in Rats

For *in vivo* absorption studies, formulations were prepared by mixing aliquots of 1% (*w*/*v*) mPEG–dendrimer with LMWH (5 mg/ml). Each 100 μl of formulation used in absorption studies contained an amount of LMWH (0.5 mg) equivalent to 30 U of anti-factor Xa activity. The formulations for subcutaneous administration contained 15 U of anti-factor Xa activity in each 100 μl of solution. The pulmonary absorption studies were carried out as described previously (17). After pulmonary and subcutaneous administration, blood samples (about 300 μl) were collected from the tip of the tail at 0, 0.5, 1, 2, 4, 8, and 12 h in citrated microcentrifuge tubes and placed on ice. Subsequently, the plasma was treated and analyzed as described previously (13–17).

Measurement of Anti-factor Xa Activity

Anti-factor Xa activity present in blood samples was determined by colorimetric assay using the Chromogenix Coatest Heparin Kit®. The assay was performed according to the protocol supplied by the manufacturer. The principle of this assay is based on the fact that the anticoagulant effect of heparin, both unfractionated and low molecular weight heparin, is achieved via their ability to bind antithrombin and inhibit clotting activity of factor-Xa. In fact, this assay uses a pharmacodynamic response to estimate indirectly absorption and bioavailability of LMWH and has been widely used to monitor therapeutic efficacy of LMWHs. In the first step of the assay, heparin forms a complex with antithrombin and the resultant complex is incubated with an excess of factor-Xa. The amount of factor-Xa neutralized by the heparin–antithrombin complex is proportional to the available amount of heparin. The remaining amount of factor-Xa hydrolyzes the chromogenic peptide substrate (S-2222) thus liberating the chromophoric group, paranitroaniline (pNA). After stopping the reaction with acetic acid, the intensity of the color is measured spectrophotometrically (405 nm). The anti-factor Xa activity was measured from the calibration curve of absorbance *vs* LMWH standard concentrations.

Efficacy of LMWH Micelles for the Treatment of Deep Vein Thrombosis

The efficacy of LMWH entrapped in mPEG–dendrimer micelles in preventing DVT was studied in a rodent model. A rat model of jugular vein thrombosis was developed according to our previously published procedure (17). Five groups of rats (six rats per group) with thrombus received the following formulations via the pulmonary route for 7 days: (a) saline with no drug once daily, (b) 100 U/kg LMWH plain drug in saline once daily, (c) 100 U/kg LMWH plain drug in saline once every 48 h, and (d) 100 U/kg LMWH in 1% mPEG–PAMAM dendrimer once every 48 h. An additional group of rats that received 50 U/kg LMWH via the subcutaneous route once daily was used as a positive control. On the eighth day of the treatment, animals were reanesthetized and the incision was reopened to inspect the jugular vein. The jugular vein was excised, and the thrombus was meticulously extracted from the vein and weighed. After extraction of the thrombus, the animals were euthanized. All animal studies were approved by the Texas Tech University Health Sciences Center (TTUHSC) Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Data Analysis

Standard noncompartmental analysis (Kinetica®, Version 4.0, Innaphase Corp. Philadelphia, PA) was performed for LMWH absorption profiles. The area under the plasma concentration *versus* time curve ($AUC_{0\rightarrow\infty}$) was calculated by the trapezoidal method. The relative bioavailability was estimated by comparing the $AUC_{0\rightarrow\infty}$ for LMWH after pulmonary delivery with that of subcutaneously administered LMWH. Data were compared by paired *t*-test or one-way ANOVA. When the differences in the means were significant, post-hoc pair wise comparisons were conducted using Newman–Keuls multiple comparison (GraphPad Prism, version 3.03, GraphPad Software, San Diego, CA). Differences in *p*-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Synthesis and Characterization of Pegylated Dendrimers

To prepare pegylated dendrimers, PEG was first activated via a reaction with 4-nitrophenyl chloroformate chloride. The hydroxyl groups of methylated PEG reacted with the reagent and eliminated a molecule of HCl as depicted in Fig. 1. Pegylation was then performed by linking activated 4-nitrophenyl chloroformate PEG (4-NPC-PEG) with G3 PAMAM dendrimer. The chemical structure of both activated mPEG and pegylated dendrimers was confirmed by FTIR and ^{1}H NMR (Figs. 2 and 3). Thin layer chromatography (TLC) was performed to determine the purity of the final pegylated dendrimer (Fig. 4). The IR spectrum of mPEG shows a major peak at 1,094 cm^{-1} that belongs to the repeating ether linkage (–C–O–C–) present in a PEG molecule. The band at 1,775 cm^{-1} in the IR spectrum of 4-nitrophenyl chloroformate chloride (NPC) is because of –C=O stretching of the carboxyl group.

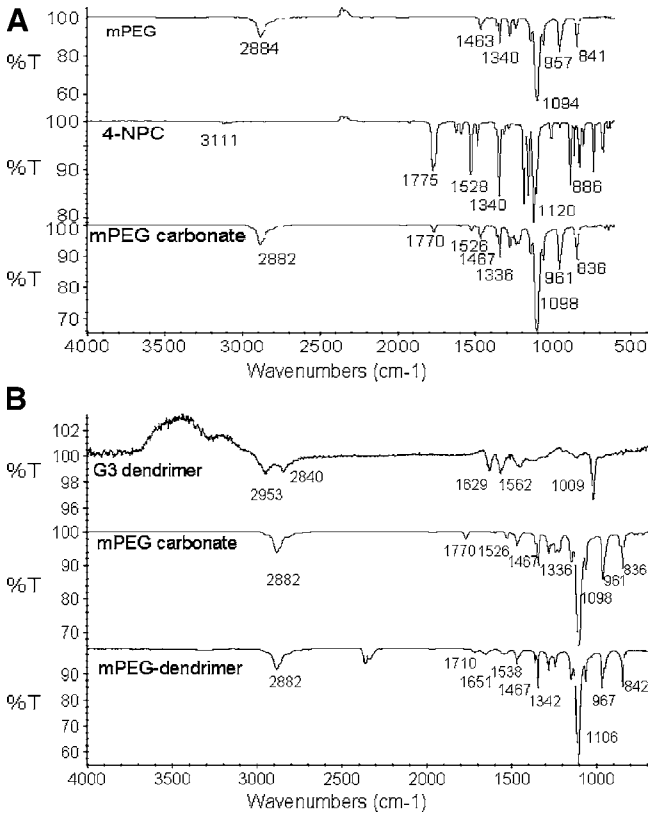

Fig. 2. FTIR spectra of (**A**) 4-nitrophenyl carbonate mPEG-2000 and (**B**) mPEG–dendrimer.

However, compared to the IR spectra of mPEG-2000 and 4-NPC, a slight shift in the bands for the –C–O–C– and –C=O functional groups was observed in the IR spectrum of activated 4-nitrophenyl chloroformate PEG. The peaks for the –C–O–C– and –C=O groups present in activated 4-nitrophenyl chloroformate PEG were observed at 1,098 and 1,770 cm^{-1}, respectively (Fig. 2A). On the other hand, the spectrum of mPEG–dendrimer shows two bands at 1,710 and 1,651 cm^{-1} that can be attributed to –C=O stretching of the carboxyl groups in the mPEG segment and amide groups in dendrimer segment, respectively, of PEG–dendrimer complex (Fig. 2B).

Similar to the IR data, ^{1}H NMR analysis also suggest that a PEG–dendrimer complex was formed upon reaction between activated PEG and G3 PAMAM dendrimer. The doublets observed at 9.6 and 8.7 ppm in the NMR scan of activated PEG suggests the presence of a benzyl ring of the 4-NPC molecule (Fig. 3A). The peak at 5.7 ppm is due to the protons of the $–CH_2OOCO–$ group of the carbonate–PEG bond, and the multiple strong peaks observed at 4.8–5.0 ppm belong to hydrogens of the repeating $–OCH_2O–$ group of PEG. The peak for the terminal $–CH_3$ group was observed at 4.6 ppm (Fig. 3A). The chemical shifts observed in the NMR spectrum of mPEG–dendrimer compound ranged between 2 and 4 ppm because protons of both the dendrimer and mPEG segment belong to $–CH_2$ and $–CH_3$ groups. The chemical shift for the protons of the CH_2 group in the dendrimer ranged from 2.2 to 2.7 ppm, and the very strong multiple peaks at 3.0–4.0 ppm belong to hydrogens of the repeating $–CH_2O–$ and terminal $–CH_3$ groups of the mPEG segment of the complex (Fig. 3B). The data presented in Figs. 2 and 3 agree with the previously published IR and NMR spectra of activated PEG and PEG–dendrimer complex, although the peak positions were slightly different (18,19).

The purity of the PEG–dendrimer compound was studied by thin layer chromatography (TLC). The TLC plates show spots for all the compounds used in the synthesis as well as that for PEG–dendrimer complex. The presence of a single spot after spraying of PEG–dendrimer complex suggests that the product was pure and free from any reactants or synthetic intermediates (Fig. 4). Furthermore, the number of amino groups substituted by activated mPEG was calculated from the integrated ratio of the NMR signal at 3.5–3.7 ppm for the proton of the $–CH_2$ group next to carbonyl groups of PAMAM and the signal at 3.3 ppm for the protons of the terminal $–OCH_3$ group of mPEG (18,19). The calculated peak ratio shows that 31.5 mPEG arms were conjugated to each dendrimer molecule, indicating that 99% of the primary surface amino groups of a dendrimer molecule were conjugated with mPEG arms. A barium-iodine assay was performed to further confirm the number of amino groups that reacted with PEG (26,17). The data from this assay (data not shown) agree with those calculated from the NMR spectra and suggest that 31.3 PEG arms were conjugated to each dendrimer molecule, and therefore 99% of the surface amino groups were covered with mPEG arms. In fact, it has previously been shown that as much as 99% of the amino groups can be linked to mPEG, resulting in almost complete pegylation of dendrimeric surface amino groups (19).

Overall, the data presented in Figs. 2, 3 and 4 suggest that an mPEG–G3–PAMAM dendrimer complex was formed upon reaction of activated PEG with dendrimers. A highly purified complex was obtained when the molar ratio of mPEG to amino groups of dendrimer was 2:1, as observed in the TLC study. Of the 32 amino groups of G3 dendrimer, 99% of them were pegylated by the methods described above. In this study we have conjugated generation 3 (G3) PAMAM dendrimer with mPEG-2000 in order to develop an inhalable formulation of LMWH with a long circulation time. Previously we have shown that G3 PAMAM dendrimer increased the pulmonary absorption of LMWH (17) but failed to prolong the circulation life of the drug. Moreover, G3 PAMAM dendrimer has been extensively studied as a drug delivery carrier because of its better safety record compared to higher generation dendrimer. In fact, although higher generation dendrimers are able to entrap large amounts of guest molecules, they have only limited *in vivo* application because of safety concerns (9). Furthermore, previous studies using pegylated particulate carriers suggest that PEG-2000 produces a maximum increase in the circulation life of drug entrapped in pegylated carriers. Compared to PEG-2000, longer-chain PEGs, including PEG-5000, have significantly shorter circulation times and a higher uptake by the liver (21). It has also been suggested that the integrity of micelles may get disrupted with the increase in chain length of PEG (18). In fact, when the molecular weight of PEG was increased from 2,000 to 5,000 Da, no change in entrapment efficiency was observed (18). It is therefore reasonable to assume that incorporation of LMWH in the micelles of mPEG-2000–G3 PAMAM dendrimers can safely and efficiently increase the circulation time of the drug.

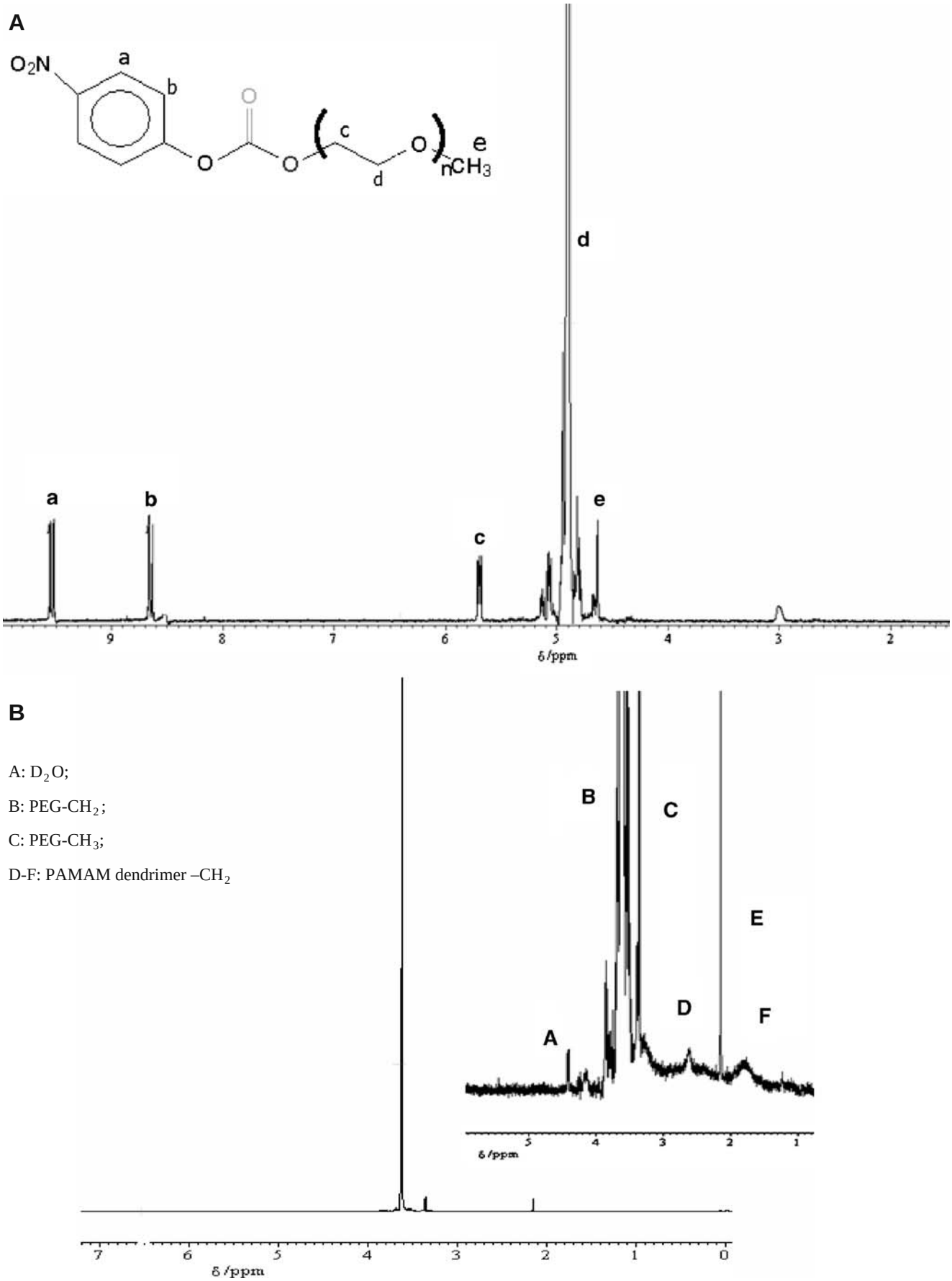

Fig. 3. NMR spectra of (**A**) 4-nitrophenyl carbonate mPEG-2000 and (**B**) mPEG–dendrimer.

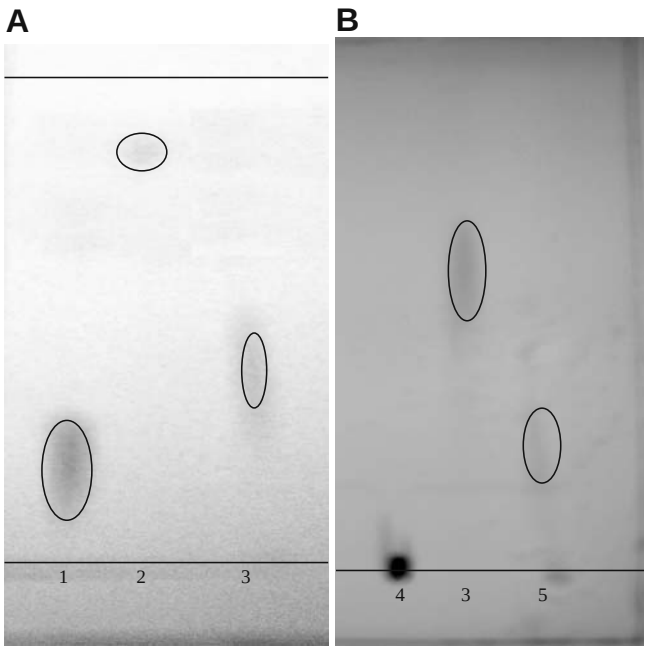

Fig. 4. Thin layer chromatography of reagents and products involved in mPEG synthesis (*1*) mPEG-2000, (*2*) 4-nitrophenyl chloroformate, (*3*) 4-nitrophenyl carbonate mPEG, (*4*) G3 PAMAM dendrimer, (*5*) mPEG–dendrimer.

Particle Size Determination of LMWH in mPEG–Dendrimer Micelles

Particle size of LMWH loaded in pegylated dendrimeric micelles was analyzed by photon spectroscopy with a NICOMP™ 380 ZLS. The particle size of plain G3 PAMAM dendrimer was not detectable by the particle sizing machine, suggesting that the size of the unimolecular micelle of dendrimer was below 5 nm, the minimum size our particle analyzer can measure. The size data for G3 PAMAM dendrimers (~3.6 nm) shown in Fig. 5 were determined by size exclusion chromatography as reported by Tomalia *et al.* (22). Upon pegylation, the particle size of the dendrimers increased from 3.6 to 11.8 nm, about a fourfold increase compared to nonpegylated dendrimer. A further increase in particle size (17.1±3.3 nm) was observed when LMWH was complexed with the PEG–dendrimer micelles (Fig. 5). In all cases, the differences in particle sizes of the three nanoconfigurations of dendrimers were statistically significant ($p<0.05$). These changes in particle size of G3 PAMAM dendrimers upon pegylation and complexation with LMWH agree with published reports. For example, size exclusion chromatographic determination of particle size showed that the hydrodynamic diameter of G3 PAMAM dendrimers and mPEG-2000–G3 dendrimers was about 3.6 and 12.5 nm, respectively (22). Furthermore, transmission electron microscopic data also suggest that the particle size of dendrimers increases upon coating with PEGs (20). In the present study, an increase in particle size of PEG–G3 dendrimer suggests that dendrimer was successfully conjugated with mPEG-2000. The observed further increase in particle size of LMWH–PEG–dendrimer complex is indicative of the drug becoming entrapped in the micelles of pegylated carriers. The increase

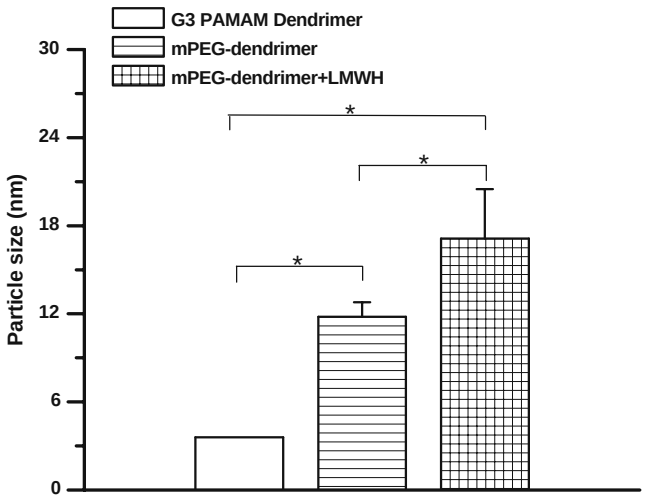

Fig. 5. Particle size of plain dendrimer and drug-entrapped mPEG–dendrimer. Data represent mean±SD, $n=3$. *Data are significantly different, $p<0.05$.

in particle size may be because a segment of linear LMWH molecule was encapsulated or interacted with the dendrimers and the other segment was left protruding outside the micelle. It is also possible that two PEG–dendrimer molecules were required to encapsulate one molecule of LMWH. A similar increase in the particle size of drug–dendrimer complex was observed when pegylated dendrimer was complexed with negatively charged DNA (23).

LMWH Loading in mPEG–Dendrimer Micelles

The azure A assay was performed to study the interaction between pegylated dendrimers and LMWH. Previously we have used the azure A assay to study electrostatic interactions between negatively charged LMWH and positively charged G3 PAMAM dendrimers (17). The data presented in Fig. 6 show that when the concentration of pegylated-dendrimer was increased from 0.25% to 1%, there

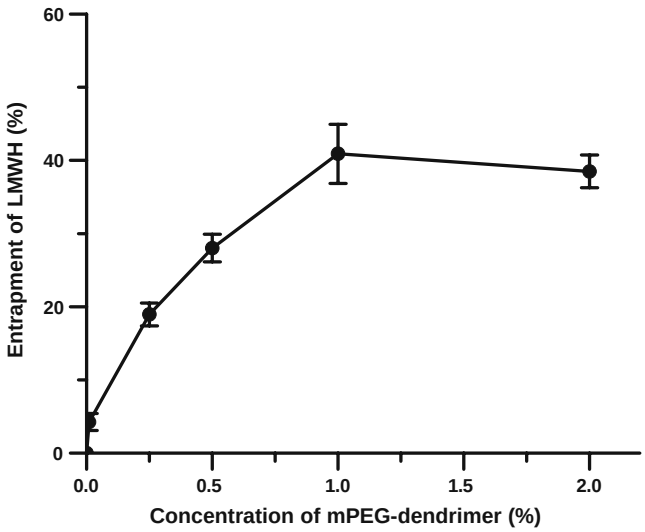

Fig. 6. Entrapment efficiency of LMWH in mPEG–dendrimer micelles. Data represent mean±SD, $n=3$–5.

was a near-linear increase in the percent entrapment efficiency of LMWH. However, with a further increase in the concentration of dendrimers, from 1% to 2%, no additional increase in drug entrapment was observed. The maximal entrapment observed was about 41%, beyond which the curve tended to flatten out (Fig. 6). Similar encapsulation patterns were observed when adriamycin and methotrexate, two anticancer drugs, were loaded in pegylated PAMAM dendrimers (19). Likewise, initially a linear increase in the solubility of a poorly soluble drug, pyrene, was observed in response to increasing concentrations of pegylated dendrimers. However, with the further increase in dendrimer concentration, a corresponding increase in the solubility was not observed (18).

The initial increase in the entrapment efficiency of LMWH was because of electrostatic interactions and/or hydrogen bonding of the drug with the dendrimeric core and PEG arms, respectively. Pegylated dendrimers form a unimolecular micelle with a hydrophilic shell around a core of dendrimer molecules. Thus, drug molecules can be entrapped both in the dendrimeric core and the hydrophilic shell of the micelles. Hydrophobic agents such as adriamycin, artemether, methotrexate, rifampicin and pyrene, are reported to interact with the hydrophobic dendritic core (18–20,24–26). Because LMWH is an excessively hydrophilic drug with a large number of carboxylic acid groups, it is reasonable to assume that complexation between LMWH and PEG–dendrimer may involve both hydrophilic and electrostatic interactions. With regard to the reduction in entrapment efficiency with the increase in PEG–dendrimer concentration, it has been argued that at higher concentrations, dendrimeric micelles may undergo aggregation, leading to a reduction in the entrapment efficiency toward the guest molecules. Yang *et al*. (18) hypothesized that with the increase in concentration of pegylated dendrimers, mPEG arms interpenetrate with each other and occupy some of the room of the inner cavities of the dendrimers. Such entanglements may prevent guest molecules like LMWH from interacting with dendrimers as the concentration of unimolecular micelles of pegylated dendrimer increases. The interpenetration of PEG arms may also disrupt the electrostatic interactions between the drug and dendrimer molecules.

In fact, drug loading in dendrimeric micelles depends on several factors, including generation of dendrimer, molecular weight of mPEG, and physicochemical properties of the drug. For example, an increase in drug loading was observed when adriamycin and methotrexate were encapsulated in pegylated G3 and G4 dendrimer. Similarly, these two drugs were encapsulated to varying degrees depending on the chemical makeup of the drug. G4 dendrimer attached to PEG-2000, for instance, can encapsulate 6.5 adriamycin molecules, 26 methotrexate molecules, and 0.4 Bengal rose molecules (19,20,27). The data presented in the present paper suggest that maximum entrapment occurred when the molar ratio of LMWHs to mPEG-2000–G3 PAMAM was 0.8, meaning that more than one PEG–dendrimer molecule was required to encapsulate a molecule of LMWH. Although loading of LWMH was found to be lower compared to the levels previously reported for smaller molecular weight drugs, this study is first to report that negatively charged LMWH with a molecular weight of 5,000 Da can be encapsulated in mPEG-conjugated G3 PAMAM dendrimers.

Pulmonary Absorption Studies in Rats

The pharmacokinetics of LMWH and LMWH encapsulated in mPEG–dendrimer was studied in a rodent model. The formulations were administered intratracheally to anesthetized rats using a MicroSprayer® (Penn-Century Inc. Philadelphia, PA) and absorption of LMWH was assessed by measuring plasma anti-factor Xa activity as described earlier (16,17). When plain LMWH was administered via the pulmonary route, an appreciable increase in anti-factor Xa activity was observed. During the first 3 h, the plasma anti-factor Xa activity was above the therapeutic level (C_{max}> 0.2 U/ml) as observed previously (17). However, the plasma level of anti-factor Xa started to decline after reaching C_{max} at 1 h and reduced to sub-therapeutic levels starting from hour 3 to the end of the 12-h study. The half-life of plain LMWH administered via the pulmonary route was also slightly shorter than that of subcutaneously administered LMWH (Fig. 7 and Table 1). The pharmacokinetics of LMWH encapsulated in mPEG–dendrimer was significantly different compared to that of plain LMWH administered via the subcutaneous or pulmonary routes. Pulmonary administered LMWH–mPEG–dendrimer formulations showed a reduced C_{max}, a longer half-life, and higher bioavailability compared to plain LMWH. Compared to the control formulation, there was about a twofold increase in $t_{1/2}$ and threefold increase in $F_{relative}$. Furthermore, the plasma anti-factor Xa levels for LMWH entrapped in mPEG–dendrimer were above the therapeutic levels for about 8 h, which is a significant improvement compared to plain LMWH.

The data in Fig. 7 and Table 1 clearly suggest that LMWH entrapped in micelles of PEG–dendrimer complex was biologically active and that this formulation approach successfully increased both the circulation time and bioavailability of the formulations. There may be several factors influencing the pharmacokinetics of LMWH encapsulated in mPEG–dendrimer complex. The initial increase in absorption

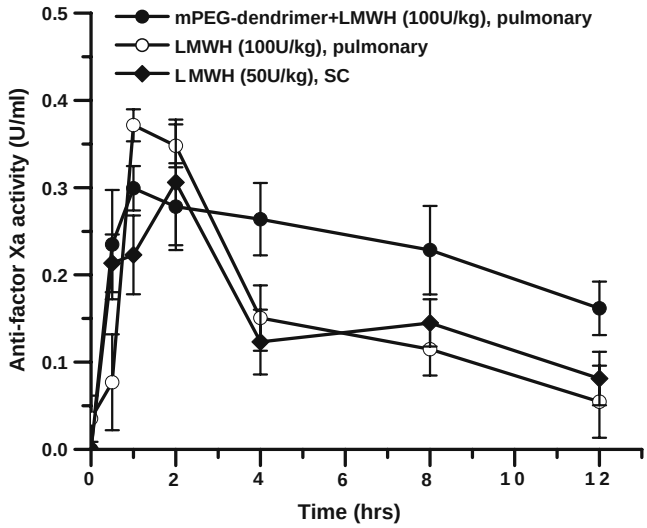

Fig. 7. Changes in anti-factor Xa activity after pulmonary or subcutaneous administration of LMWH formulated in saline or micelles. Data represent mean±SD, n=3–5.

Table 1. Pharmacokinetic Parameters Following Pulmonary (100 U/kg) and Subcutaneous (50 U/kg) Administration of LMWH Formulations

Formulation	C_{max} (U/ml)	$t_{1/2}$ (h)	T_{max} (h)	$AUC_{0\rightarrow\infty}$ (U·h/ml)	$F_{relative}$ (%)
LMWH saline	0.36±0.03	4.9±3.3	1.5±0.6	2.0±0.5	21.7±4.9
LWMH micelles	0.29±0.03	11.9±2.7*	1.5±0.6	5.7±1.8*	60.6±19.1*
LMWH saline SC	0.32±0.08	6.5±1.8	2.0±0.0	4.7±1.3	–

Data represent mean±SD, n=3–5
*p<0.05 compared to LMWH saline group

was because of free drug that was not entrapped in the formulation. As observed in the azure A assay (Fig. 6), about 60% of the drug was available in free form in the pegylated dendrimers and similar to plain LMWH; this free drug was absorbed via passive diffusion across the pulmonary epithelium. Compared to plain pulmonary LMWH, the C_{max} for LMWH formulated in mPEG–dendrimer was lower perhaps because the entire dose of the drug was not available for immediate absorption. However, release and absorption of the drug entrapped in the nanocarriers are influenced by the type of drug–dendrimer interaction, the size of micelles containing the drug, and the partitioning of the drug and carrier in biological membranes. One of the hypotheses for slower absorption of drug encapsulated in mPEG–dendrimer micelles is that micelles containing drug remain in a dynamic equilibrium with free drug and free micelles in lung fluids. As the drug diffuses via the respiratory epithelium, more drug molecules are released from the complex and the process of drug release and absorption continues until all drug is absorbed or eliminated from the respiratory system. This hypothesis rules out the possibility of absorption of intact drug–dendrimer complex. It is also possible that pegylation increases entrapment efficiency and so more drug is available for absorption from pegylated dendrimers compared to nonpegylated dendrimers. Alternatively, drug entrapped in pegylated dendrimers may undergo endocytosis in the respiratory epithelium as is observed in the case of DNA–dendrimer complexes. This hypothesis is based on the fact that, similar to DNA, LMWH forms electrostatic complexes with dendrimers because of its negative surface charge. In fact, it has been reported that the presence of PEG surface moieties increases the transfection efficiency of dendrimer–DNA complex (28). Like DNA–dendrimer complex, intact LMWH–dendrimer complex could be endocytosed through pulmonary cell membranes before the drug is finally released to the circulation. If the assumption of endocytosis is true, then the complex must be avoiding recognition by cells of reticuloendothelial systems (RES), presumably because of the hydrophilic shell of PEG moieties around the hydrophobic core of the dendrimers and the particle being submicronic in size. When the particle size of a drug-containing carrier is smaller than 100 nm, RES cannot readily recognize those particles. The particle size of the proposed formulation of LMWH in mPEG–dendrimer was in fact below 100 nm (Fig. 5). Therefore, both PEG shielding and small size of the particles may have contributed to the drug's long circulation. This observation also agrees with previously published data that showed the pegylated dendritic micelles produce sustained release both *in vitro* and *in vivo* (19,20,29).

Therapeutic Efficacy of LMWH–mPEG–Dendrimer in the Treatment of Deep Vein Thrombosis

The efficacy of LMWH encapsulated in mPEG–dendrimer micelles in preventing DVT was tested in a rat jugular vein thrombosis model. We and others have previously used this model to study the antithrombotic effect of LMWH (17,30,31). Formulations were administered via the pulmonary route and the weight of thrombus formed in the rat jugular vein was recorded to assess the efficacy of the formulation. The thrombus weight in rats that received no drug but saline was 5.3±0.7 mg. The amount of thrombus in rat jugular vein was drastically reduced from 5.3±0.7 mg to 0.5±0.5 mg in rats that received subcutaneous LMWH at a dose of 50 U/kg once daily (Fig. 8). Similar to subcutaneous LMWH, when rats received a once-daily dose of 100 U/kg LMWH via the pulmonary route, the thrombus weight was reduced from 5.3±0.7 mg to 0.8±0.8 mg, about an 85%

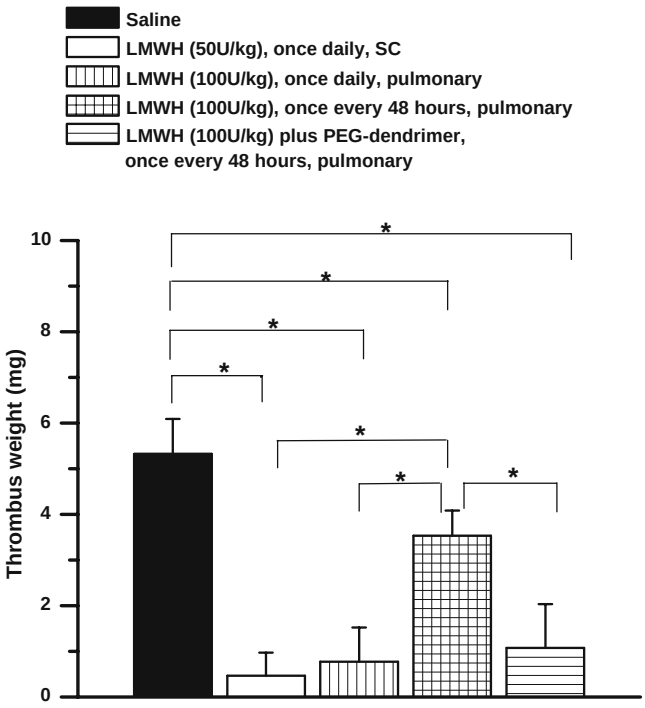

Fig. 8. Efficacy of LMWH plus mPEG–dendrimer in the treatment of jugular vein thrombosis. Data represent mean±SD, n=3–5. *Results are significantly different, p<0.05.

reduction compared to the saline control group. The differences between thrombus weight in rats treated with subcutaneous LMWH and those treated with pulmonary LMWH were not statistically significant ($p>0.05$). The data in Fig. 8 also suggest that 100 U/kg of pulmonary LMWH was as efficacious as 50 U/kg subcutaneous LMWH. However, when the dosing interval for pulmonary administration was increased from 24 to 48 h, the thrombus weight in rats that received plain LMWH was reduced to 3.5±0.6 mg, a 34% reduction compared to the saline control. Although the thrombus weight in this treatment group was lower than that of the saline-treated rats, it was still higher compared to that observed in rats treated with a once-daily dose of 100 U/kg LMWH administered via the pulmonary route. When rats received LMWH encapsulated in mPEG–dendrimer via the pulmonary route every 48 h for 7 days, the thrombus weight was reduced by 79% compared to that of the saline control and by 68% compared to that of rats receiving plain aerosolized LMWH administered every 48 h. Furthermore, the extent of reduction in thrombus weight produced by LMWH–mPEG–dendrimer formulation administered at 48-h intervals was very similar to that observed in rats treated with subcutaneous and aerosolized plain LMWH administered once daily ($p>0.05$). In other words, LMWH–mPEG–dendrimer formulation administered via the pulmonary route every 48 h was as efficacious as subcutaneous LMWH administered at 24-h intervals. Importantly, although there was an increase in dosing interval, LMWH entrapped in micelles was effective in eliminating the thrombus formed in rat jugular vein. These data agree with the absorption profiles presented above (Fig. 7), which showed an increase in the half-life of anti-factor Xa for LMWH entrapped in mPEG–dendrimer micelles. The longer $t_{1/2}$ produced an increase in the duration of the anti-coagulant effect of LMWH, which is evident from the data presented in Fig. 8. Although plain LMWH with a relative bioavailability of 21.7% produced a therapeutic effect, it failed to maintain a therapeutic level of anti-factor Xa for 2 days. However, LMWH-entrapped in micelles with a bioavailability of 61% was effective in maintaining a therapeutic concentration and anti-coagulant effect for more than 2 days. Therefore, this formulation is considered to be long-circulating and likely to reduce the dosing frequency.

On the whole, this study is the first to show that mPEG–dendrimer can be used for pulmonary delivery of LMWH. An increase in entrapment efficiency was observed when the concentration of PEG–dendrimer was increased from 0.25% to 1%. Upon encapsulation of LMWH, an increase in particle size was also observed. This study also shows that the half-life and absorption of LMWH administered via the pulmonary route can be increased by encapsulating the drug in dendrimeric micelles. This formulation strategy is likely to reduce the dosing frequency from once daily to once every other day and maintain the therapeutic efficacy of LMWH for 48 h. Importantly, this study suggests that LMWH loaded in the mPEG–dendrimer could potentially be used as noninvasive delivery system for the treatment of thromboembolic disorder. However, caution should be exercised in using the formulation in a clinical setting. Although dendrimeric nanocarrier has spurred an interest in drug delivery, data on the safety of the carrier is still very limited. Further studies should be performed to determine the safety of the formulation upon repeated administration of the formulations via the lungs.

ACKNOWLEDGEMENT

This study was supported by NIH R15 HL7713302.

REFERENCES

1. K. T. Al-Jamal, C. Ramaswamy, and A. T. Florence. Supramolecular structures from dendrons and dendrimers. *Adv Drug Deliv Rev.* **57**:2238–2270 (2005). doi:10.1016/j.addr.2005.09.015.
2. A. D'Emanuele, and D. Attwood. Dendrimer–drug interactions. *Adv Drug Deliv Rev.* **57**:2147–2162 (2005). doi:10.1016/j.addr.2005.09.012.
3. C. Dufes, I. F. Uchegbu, and A. G. Schatzlein. Dendrimers in gene delivery. *Adv Drug Deliv Rev.* **57**:2177–2202 (2005). doi:10.1016/j.addr.2005.09.017.
4. R. Duncan, and L. Izzo. Dendrimer biocompatibility and toxicity. *Adv Drug Deliv Rev.* **57**:2215–2237 (2005). doi:10.1016/j.addr.2005.09.019.
5. A. T. Florence, and N. Hussain. Transcytosis of nanoparticle and dendrimer delivery systems: evolving vistas. *Adv Drug Deliv Rev.* **50**:S69–89 (2001). doi:10.1016/S0169-409X(01)00184-3.
6. K. M. Kitchens, M. E. El-Sayed, and H. Ghandehari. Transepithelial and endothelial transport of poly (amidoamine) dendrimers. *Adv Drug Deliv Rev.* **57**:2163–2176 (2005). doi:10.1016/j.addr.2005.09.013.
7. H. Kobayashi, and M. W. Brechbiel. Nano-sized MRI contrast agents with dendrimer cores. *Adv Drug Deliv Rev.* **57**:2271–2286 (2005). doi:10.1016/j.addr.2005.09.016.
8. S. Svenson, and D. A. Tomalia. Dendrimers in biomedical applications—reflections on the field. *Adv Drug Deliv Rev.* **57**:2106–2129 (2005). doi:10.1016/j.addr.2005.09.018.
9. S. Bai, C. Thomas, A. Rawat, and F. Ahsan. Recent progress in dendrimer-based nanocarriers. *Crit Rev Ther Drug Carrier Syst.* **23**:437–495 (2006).
10. J. C. Roberts, M. K. Bhalgat, and R. T. Zera. Preliminary biological evaluation of polyamidoamine (PAMAM) Starburst dendrimers. *J Biomed Mater Res.* **30**:53–65 (1996). doi:10.1002/(SICI)1097-4636(199601)30:1<53::AID-JBM8>3.0.CO;2-Q.
11. S. M. Moghimi, A. C. Hunter, and J. C. Murray. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol Rev.* **53**:283–318 (2001).
12. Y. Qi, G. Zhao, D. Liu, Z. Shriver, M. Sundaram, S. Sengupta, G. Venkataraman, R. Langer, and R. Sasisekharan. Delivery of therapeutic levels of heparin and low-molecular-weight heparin through a pulmonary route. *Proc Natl Acad Sci U S A.* **101**:9867–9872 (2004). doi:10.1073/pnas.0402891101.
13. T. Yang, J.J. Arnold, and F. Ahsan. Tetradecylmaltoside (TDM) enhances *in vitro* and *in vivo* intestinal absorption of enoxaparin, a low molecular weight heparin. *J Drug Target.* **13**:29–38 (2005). doi:10.1080/10611860400020191.
14. T. Yang, A. Hussain, S. Bai, I. A. Khalil, H. Harashima, and F. Ahsan. Positively charged polyethylenimines enhance nasal absorption of the negatively charged drug, low molecular weight heparin. *J Control Release.* **115**:289–297 (2006). doi:10.1016/j.jconrel.2006.08.015.
15. T. Yang, F. Mustafa, and F. Ahsan. Alkanoylsucroses in nasal delivery of low molecular weight heparins: *in-vivo* absorption and reversibility studies in rats. *J Pharm Pharmacol.* **56**:53–60 (2004). doi:10.1211/0022357022377.
16. T. Yang, F. Mustafa, S. Bai, and F. Ahsan. Pulmonary delivery of low molecular weight heparins. *Pharm Res.* **21**:2009–2016 (2004). doi:10.1023/B:PHAM.0000048191.69098.d6.
17. S. Bai, C. Thomas, and F. Ahsan. Dendrimers as a carrier for pulmonary delivery of enoxaparin, a low-molecular weight heparin. *J Pharm Sci.* **96**:2090–2106 (2007). doi:10.1002/jps.20849.
18. H. Yang, J. J. Morris, and S. T. Lopina. Polyethylene glycol-polyamidoamine dendritic micelle as solubility enhancer and the effect of the length of polyethylene glycol arms on the solubility

of pyrene in water. *J Colloid Interface Sci.* **273**:148–154 (2004). doi:10.1016/j.jcis.2003.12.023.
19. C. Kojima, K. Kono, K. Maruyama, and T. Takagishi. Synthesis of polyamidoamine dendrimers having poly(ethylene glycol) grafts and their ability to encapsulate anticancer drugs. *Bioconjug Chem.* **11**:910–917 (2000). doi:10.1021/bc0000583.
20. D. Bhadra, S. Bhadra, S. Jain, and N. K. Jain. A PEGylated dendritic nanoparticulate carrier of fluorouracil. *Int J Pharm.* **257**:111–124 (2003). doi:10.1016/S0378-5173(03)00132-7.
21. K. Maruyama, T. Yuda, A. Okamoto, S. Kojima, A. Suginaka, and M. Iwatsuru. Prolonged circulation time *in vivo* of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). *Biochimica et Biophysica Acta.* **1128**:44–49 (1992).
22. D. A. Tomalia, A. M. Naylor, and W. A. Goddard III. Starburst dendrimers: molecular-level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter. *Angew Chem, Int Ed Engl.* **29**:138–175 (1990). doi:10.1002/anie.199001381.
23. J. S. Choi, E. J. Lee, Y. H. Choi, Y. J. Jeong, and J. S. Park. Poly (ethylene glycol)-block-poly(L-lysine) dendrimer: novel linear polymer/dendrimer block copolymer forming a spherical water-soluble polyionic complex with DNA. *Bioconjug Chem.* **10**:62–65 (1999). doi:10.1021/bc9800668.
24. R. Jevprasesphant, J. Penny, R. Jalal, D. Attwood, N. B. McKeown, and A. D'Emanuele. The influence of surface modification on the cytotoxicity of PAMAM dendrimers. *Int J Pharm.* **252**:263–266 (2003). doi:10.1016/S0378-5173(02)00623-3.
25. M. Liu, K. Kono, and J. M. Frechet. Water-soluble dendritic unimolecular micelles: their potential as drug delivery agents. *J Control Release.* **65**:121–131 (2000). doi:10.1016/S0168-3659(99)00245-X.
26. G. Pan, Y. Lemmouchi, E. O. Akala, and O. Bakare. Studies on PEGylated and drug-loaded PAMAM dendrimers. *J Bioact Compat Polym.* **20**:113–128 (2005). doi:10.1177/0883911505049656.
27. Y. Haba, C. Kojima, A. Harada, T. Ura, H. Horinaka, and K. Kono. Preparation of poly(ethylene glycol)-modified poly(amido amine) dendrimers encapsulating gold nanoparticles and their heat-generating ability. *Langmuir.* **23**:5243–5246 (2007). doi:10.1021/la0700826.
28. R. Q. Huang, Y. H. Qu, W. L. Ke, J. H. Zhu, Y. Y. Pei, and C. Jiang. Efficient gene delivery targeted to the brain using a transferrin-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. *FASEB J.* **21**:1117–1125 (2007). doi:10.1096/fj.06-7380com.
29. T. Ooya, J. Lee, and K. Park. Effects of ethylene glycol-based graft, star-shaped, and dendritic polymers on solubilization and controlled release of paclitaxel. *J Control Release.* **93**:121–127 (2003). doi:10.1016/j.jconrel.2003.07.001.
30. K. Salartash, M. D. Gonze, A. Leone-Bay, R. Baughman, W. C. Sternbergh 3rd, and S. R. Money. Oral low-molecular weight heparin and delivery agent prevents jugular venous thrombosis in the rat. *J Vasc Surg.* **30**:526–531 (1999). doi:10.1016/S0741-5214(99)70080-7.
31. M. D. Gonze, K. Salartash, W. C. Sternbergh 3rd, R. A. Baughman, A. Leone-Bay, and S. R. Money. Orally administered unfractionated heparin with carrier agent is therapeutic for deep venous thrombosis. *Circulation*. **101**:2658–2661 (2000).