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Research Paper

Complexation of a Poly-L-Arginine with Low Molecular Weight Heparin Enhances Pulmonary Absorption of the Drug

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Purpose. This study tests the hypothesis that complexation between a cationic polymer, poly-L-arginine (PLA), and an anionic drug, low molecular weight heparin (LMWH), enhances pulmonary absorption and reduces the epithelial toxicity.
Materials and Methods. Enoxaparin, a LMWH, was complexed with PLAs of different molecular weights at varying concentrations. The resulting complexes were characterized by measuring particle size and zeta potential, and by quantitating the interactions between PLA and enoxaparin using an azure A assay. Changes in transepithelial electrical resistance (TEER) and cytotoxicity induced by enoxaparin–PLA complex were investigated in Calu-3 cells. Pulmonary absorption of LMWH was determined by measuring plasma anti-factor Xa levels. A bronchoalveolar lavage (BAL) study was performed to investigate if the PLA-based formulations caused any cellular or biochemical changes in the lungs.
Results. The particle size of enoxaparin–PLA complexes decreased and the zeta potential values of the complex became less negative as the concentration of positively charged PLA in the complex increased. *In vitro* experiments suggest that addition of enoxaparin–PLA complex to the apical side of a polarized cell monolayer results in a significant increase in permeability to ^{14}C-mannitol and a decrease in TEER. Pulmonary formulations of enoxaparin plus 0.0125% or 0.0625% PLA of molecular weight 93 kDa led to a twofold increase in the relative bioavailability of LMWH compared to the control (enoxaparin plus normal saline). The BAL study showed that the enoxaparin–PLA complex formulation did not elicit any significant increases in marker enzyme activities compared to the normal saline-treated or untreated control groups.
Conclusion. PLA could be used as a carrier for the pulmonary delivery of LMWH.

KEY WORDS: enoxaparin; low molecular weight heparin; poly-L-arginine; pulmonary drug delivery.

INTRODUCTION

Poly-L-arginine (PLA) is a homopolymer of the amino acid L-arginine. Because of the positively charged guanidine group on the side chain of L-arginine, PLA is considered to be a cationic polyamino acid with a high density of positive surface charges. Homopolymers of positively charged amino acids have the unique ability to increase the permeability of the cell membrane and facilitate absorption of both large and small molecules (1–3). It has been demonstrated that PLA is transported across the cell membrane better than polymers of other cationic amino acids such as ornithine, lysine and histidine. Since PLA is a polymer of an essential amino acid, it mimics properties of endogenous proteins; it also has a relatively low toxicity on mucosal membranes and is biodegradable. These properties of PLA have made it an ideal candidate for a drug carrier and drug absorption promoter *via* intramucosal routes (4,5). The application of positively charged and amphipathic peptides, such as arginine-rich peptides, in drug delivery has recently been reviewed (6,7). Various arginine-rich peptides increase the cellular uptake of proteins, which is believed to be facilitated by the guanidinium ions of the peptides. Positively charged peptides can facilitate diffusion into the nonpolar cell membrane by interacting with negatively charged epithelial surface components. Entry of the peptides into the cell is achieved through neutralization of the epithelial membrane and transient opening of the tight junctions or by utilizing the aqueous pore (paracellular) pathway, which selectively transports hydrophilic as well as ionized compounds across the nasal epithelium (8,9).

The efficacy of PLA in enhancing nasal absorption of the hydrophilic model compound fluorescein isothiocyanate-labeled dextran (FD-4) has been studied. Ohtake *et al.* (8) showed that PLA enhances nasal absorption of FITC-dextran in a dose- and molecular weight-dependent manner. The absorption-enhancing effect of PLA was transient and reversible and was also dependent on the charge density of the PLA molecule. Changes in PLA-induced paracellular permeability

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of rabbit nasal epithelium were accompanied by serine/threonine phosphorylation of ZO-1 and tyrosine dephosphorylation of occludin (8). Furthermore, positively charged polymers are reported to increase bronchial drug absorption through endothelial cells by modulating membrane proteins and translocation (10,11).

As PLA enhances nasal absorption and epithelial permeability, it is also likely to increase pulmonary absorption of drugs that are required to be administered as injections because of poor oral bioavailability. In fact, the pulmonary route offers several advantages over the nasal route in terms of systemic delivery of molecules. These advantages include: (1) the lower extracellular enzymatic activity; (2) the large absorptive surface area (100 m^2); (3) the extensive vasculature; (4) the thin of the alveolar epithelium (0.1–0.2 μm); and (5) the short distance of the air–blood exchange pathway (12–14). Furthermore, because of the presence of a viscoelastic protective layer in the lower respiratory tract, the extent of irritation caused by a pulmonary formulation is likely to be substantially lower compared to a nasally administered drug formulation. However, to our knowledge there are no data as to the use of PLA in enhancing pulmonary absorption of large hydrophilic drugs that can be used for the treatment of pulmonary embolism. In addition to PLA's ability to increase drug absorption from the mucosal route, PLA has been reported to prevent embolism-induced lung injury by an antioxidant mechanism involving increased NO synthesis (15,16).

Low molecular weight heparins (LMWHs) are negatively charged oligosaccharides used in the treatment of deep vein thrombosis and pulmonary embolism (17–19). Unfortunately, the presence of carboxylic acid and sulfate groups in the glycosaminoglycan units of LMWH render it highly anionic and thus an unlikely candidate for absorption via the mucosal routes. Furthermore, the application of LMWH on an outpatient basis has been limited because of the requirement of daily subcutaneous injections. Consequently, attempts have been made to deliver LMWH via noninvasive routes, including the nasal and pulmonary routes (20–22). We have previously tested various formulation approaches that can be used to enhance absorption of LMWH via the respiratory tract (21, 22). However, the use of PLA in a pulmonary formulation of LMWH is yet to be established. This study was therefore designed to test the hypothesis that PLA enhances pulmonary absorption of LMWH and that the resulting LMWH-PLA complex is relatively safe for administration to the lungs. In this regard, the drug was formulated with PLA, and the safety and efficacy of the resulting formulations were tested in rodent and cell culture models.

MATERIALS AND METHODS

Materials

Poly-L-arginine (molecular weight 14 kDa, 35 kDa and 93 kDa; pKa≤3.0 at 25°C), D-[1-^{14}C] mannitol (specific activity, 53 mCi/mmol), (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and lipopolysaccharide (LPS; *E. Coli* 055:B5) were obtained from Sigma Chemical Company (St. Louis, MO). Immortalized Calu-3 cells, Modified Eagle's Medium (MEM), L-glutamine, fetal bovine serum, penicillin and streptomycin solution, and trypsin EDTA solution were purchased from ATCC (Rockville, MD). Vitrogen 100 solution was obtained from Cohesion, Inc. (Palo Alto, CA). Transwells® cell culture assembly with polycarbonate inserts (0.4 μm pore size, 1 cm^2 area) were obtained from Corning Costar Corporation (Cambridge, MA). The low molecular weight heparin Enoxaparin (Lovenox®, 3000 U of anti-factor Xa activity per 0.3 ml; LMWH) injections were obtained as sterile solutions from Aventis Pharmaceutical Products Inc. (Bridgewater, NJ). The reagent for detecting lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were purchased from Pointe Scientific Inc. (Lincoln Park, MI). *N*-acetylglucosaminidase (NAG) assay kit was obtained from Equal Diagnostics (Exton, PA). Sodium dodecyl sulphate (SDS) was purchased from Bio-rad Laboratories (Hercules, CA).

Preparation of Enoxaparin–PLA Complex Formulations

All complexes between enoxaparin and poly-L-arginine (PLA) were freshly prepared before the experiments. Prior to the preparation of the complex, the polymer and drug were diluted with normal saline. An aliquot of diluted PLA solution was added to the enoxaparin solution and mixed by vortexing for 10 s. The mixtures were then incubated for 30 min at room temperature to allow complex formation. The concentrations of PLA used in the formulations were 0.0125, 0.0625 or 0.125% (*w*/*v*). The drug concentration of the final formulation was such that each 100 μl of the solution contained an amount of enoxaparin sodium (1 mg) equivalent to 100 U of anti-factor Xa activity. Similarly, for subcutaneous absorption studies, formulations were made by diluting enoxaparin with normal saline. Formulations for subcutaneous administration were prepared to contain 100 U of anti-factor Xa activity in each 100 μl of solution.

Characterization of Enoxaparin–PLA Complexes

For the measurement of particle size and zeta potential, freshly prepared formulations containing varying concentrations (0.0125, 0.0625 or 0.125%) of three different PLAs (14, 35 or 93 kDa) were diluted in normal saline. The measurements were performed in triplicates using a NICOMP™380 ZLS, PSS-Nicomp particle sizing system (Santa Barbara, CA). For zeta potential, measurements were performed using the electrophoretic light scattering method at 23°C at a scattering angle of 14.1° and E-field strength of 10 V/cm. The viscosity and dielectric constant of deionized water were used as calculation parameters.

Quantitation of Enoxaparin–PLA Interactions

The interactions between LMWH and PLA were further tested by a colorimetric assay using azure A blue dye as described by Cadene *et al.* (23). To do this test, a fixed amount of azure A dye solution was mixed with the following test samples: (1) enoxaparin solutions containing increasing concentrations (5, 10, 20, 30, 40, 50, and 100 μM) of the drug; (2) 0.0125, 0.0625 and 0.125% (*w*/*v*) solutions of PLA; or (3) various enoxaparin–PLA formulations containing 15 U of LMWH. In each case, 50 μl (150 μM) of azure A dye was mixed with 20 μl of test sample in a 96-well microplate and the resulting mixture was incubated for 30 min at room temperature. The samples were then measured by a microtiter-plate reader (TECAN US Inc., Research Triangle Park, NC) at 595 nm.

Transport and TEER Studies across Calu-3 Cell Monolayers

Calu-3 cells were grown in MEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 37°C incubator with 5% CO_2. Calu-3 cells were plated in 75 cm^2 culture flasks (coated with fibronectin/collagen solution) and sub-cultured after achieving 85–90% confluence. The medium was changed every 2 days and the passages used for the experiment were between 8 and 15. The inserts of the Transwells® (0.4 μm pore size, 1 cm^2 area) were coated with Vitrogen 100 solution. The coating procedures were performed by adding a freshly prepared chilled solution (8 ml Vitrogen-100, 1 ml 10X PBS, 1 ml 0.1 M sodium hydroxide, adjusted to pH 7.4) to the Transwells® insert surface, then incubating at 37°C for at least 1 h to induce the *in situ* gel formation. Transwells® were then dried overnight under laminar airflow.

For transport studies, the Calu-3 cells were seeded at a density of 50,000 cells/well onto collagen-coated polycarbonate Transwells® (0.4 μm pore size, 1 cm^2 area). After the cells had attached to the Transwells®, the medium was removed from the apical compartment to allow the monolayer to grow at the air-interface. The integrity of the confluent cell monolayers was evaluated by measuring transepithelial electrical resistance (TEER) using an EVOM® epithelial voltohmeter (World Precision Instruments, Sarasota, FL). The monolayers with TEER values above 400 $\Omega \cdot cm^2$ were used in the experiments (about 10–14 days). ^{14}C-mannitol flux was determined for each set of cells used in the transport experiments.

On the day of experiment, ^{14}C-mannitol were dissolved in normal saline and mixed with enoxaparin–PLA complex solution. The final concentrations of PLA used in the experiments were 0.0125, 0.0625 and 0.125% (*w/v*). Prior to the initiation of transport experiments, 1.5 ml normal saline was added to the basolateral side and 0.5 ml of pre-warmed solutions containing ^{14}C-mannitol were added to the apical side in the presence or absence of enoxaparin–PLA complex. Monolayers for transport experiments were kept in a 37°C/5% CO_2 incubator. Samples (100 μl) were withdrawn from the basolateral chamber at various time intervals (0, 15, 30, 45, 60, 90, 120 min). The basolateral chamber was replenished with fresh medium (100 μl) after each sampling. TEER was recorded during the experiments.

The amount of ^{14}C-mannitol transported across the cell monolayers was determined by a Beckman LS 6500 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The apparent permeability coefficient (P_{app}) was calculated from the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \cdot Co}$$

Where dQ/dt is the flux of ^{14}C-mannitol across the cell monolayer (cpm/s), A is the surface area available for transport (1 cm^2 for the 12-well Transwells® system), and C_0 is the initial concentration (cpm/ml) of mannitol in the donor compartment.

MTT Cytotoxicity Studies

For the cytotoxicity studies, Calu-3 cells were seeded at a density of 50,000 cells/well in flat-bottom, 96-well microtiter tissue culture plates. Immediately prior to the start of the experiment, the medium was removed from the wells and the cells were washed with normal saline. Subsequently, the cells were incubated with 200 μl of enoxaparin–PLA-93 kDa complex for 0.5, 2, 6 and 24 h, separately. Sodium dodecyl sulfate (SDS) was used at 0.1% as a positive cytotoxic control. The cell viability was measured by the MTT assay as previously described (24). Briefly, after 0.5, 2, 6 or 24 h, MTT (5 mg/ml) solution was added to each well and the cells were incubated at 37°C for 4 h. Next, the solution in each well was removed and acidified isopropyl alcohol (100 μl of 1% v/v, concentrated hydrochloric acid in isopropyl alcohol) was added. Finally, the plates were incubated at 37°C for 1 h. Untreated Calu-3 cells were also harvested to determine the effect of enoxaparin–PLA complex or SDS on the cells. Optical density of the cells was measured on a microtiter-plate reader (TECAN US Inc.) at 570 nm. Each assay was performed on six samples and cell viability was expressed as the percentage of MTT released by cells exposed to enoxaparin–PLA complex or SDS compared to cells incubated with medium alone.

Pulmonary Absorption Studies in Rats

Male Sprague–Dawley rats (Charles River Laboratories, Charlotte, NC) weighing between 250–350 g were used for *in vivo* absorption studies (6 rats in each group). Prior to the experiment, the animals were anesthetized by intramuscular injection of a mixture containing xylazine (20 mg/ml) and ketamine (100 mg/ml). Anesthesia was maintained with additional intramuscular injections of anesthetic solution as needed throughout the experiments. Enoxaparin–PLA complex formulations (50 U/kg) were administered intratracheally as described earlier (25). The amount of formulation administered was 80–120 μl depending on animal body weight. For bioavailability studies, plain enoxaparin was administered subcutaneously (50 U/kg) as a single injection under the dorsal skin. After pulmonary and subcutaneous administration, blood samples (~300 μl) were collected from the tip of the rat-tail at 0, 15, 30, 60, 120, 240, 360, 480, 600 and 720 min in citrated microcentrifuge tubes and placed on ice. Subsequently, the plasma was separated by centrifugation (1,600×*g* for 5 min) and the plasma samples thus obtained were stored at −20°C until further analysis. Enoxaparin absorption was determined by measuring plasma anti-factor Xa levels using a colorimetric assay kit (Chromogenix Coatest Heparin Kit®, Diapharma Group Inc., West Chester, OH).

Bronchoalveolar Lavage Studies

The bronchoalveolar lavage (BAL) studies were performed according to our previously reported method (26). Rats were divided into four groups, six rats in each group, to receive four different treatments. Three groups of animals received three control formulations: sodium dodecyl sulfate (SDS, 0.1%) and lipopolysaccharide (LPS, 0.1 μg/ml) as positive controls, and normal saline as negative control. The fourth group of animals received enoxaparin complexed with the largest molecular weight PLA at the highest concentration (0.125% PLA-93K). All formulations were administered as described above and rats were re-anesthetized at 24 h after

drug administration and the respiratory apparatus was exposed by a mid-level incision in the thoracic cavity. The lungs were surgically removed after ex-sanguination by severing the abdominal aorta and wet lung weight was recorded. The lungs were lavaged by instillation of a 5-ml aliquot of cold normal saline into the trachea, left in the lungs for 30 s, withdrawn, re-instilled for an additional 30 s and finally withdrawn. The sampled fluid was centrifuged at $500\times g$ for 10 min and the supernatant was collected for analysis of lactate dehydrogenase (LDH), alkaline phosphatase (ALP) or *N*-acetylglucosaminidase (NAG) activity. The concentrations of these enzymes in the BAL fluid were determined using commercially available kits and their activities were expressed as U/ml.

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.

Pharmacokinetic Analysis

Standard pharmacokinetic analysis (Kinetica®, Version 4.0, Innaphase Corp. Philadelphia, PA) was performed for enoxaparin absorption-time profiles. Area under the plasma concentration versus time curve ($AUC_{0\rightarrow720}$) was calculated by the trapezoidal method. Relative bioavailabilities ($F_{relative}$) were estimated by comparing $AUC_{0\rightarrow720}$ for pulmonary administered enoxaparin with that of subcutaneously administered enoxaparin.

Statistical Analyses

Particle size values, permeation coefficients, TEER values, pharmacokinetic parameters and marker enzyme activities of different formulations 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), and *p* values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Characterization of Enoxaparin–PLA Complex

Enoxaparin–PLA complex was prepared by mixing the polymer with the drug at a nonstoichiometric ratio and the resulting complex, known as the polyelectrolyte complex, was characterized by measuring particle size and zeta potential (Fig. 1). Plain PLA (0.5%) or enoxaparin solution (30 U/ml) did not show the presence of particles in the solution, suggesting that both drug and polymer were completely soluble in normal saline. However, enoxaparin–PLA complex showed a particle size ranging from 100 nm to 500 nm and the particle size of the complex increased with the increasing concentration of PLA ($p<0.05$). There was no significant increase in particle size with the increase in the molecular weight of the polymer (Fig. 1a). The increase in particle size of the mixture of enoxaparin–PLA suggests that complexation has occurred, presumably through electrostatic interactions between the negatively charged drug and the positively charged polymer. As more PLA was added to a fixed amount LMWH, the particle size continued to increase because of formation of larger particles due to aggregations.

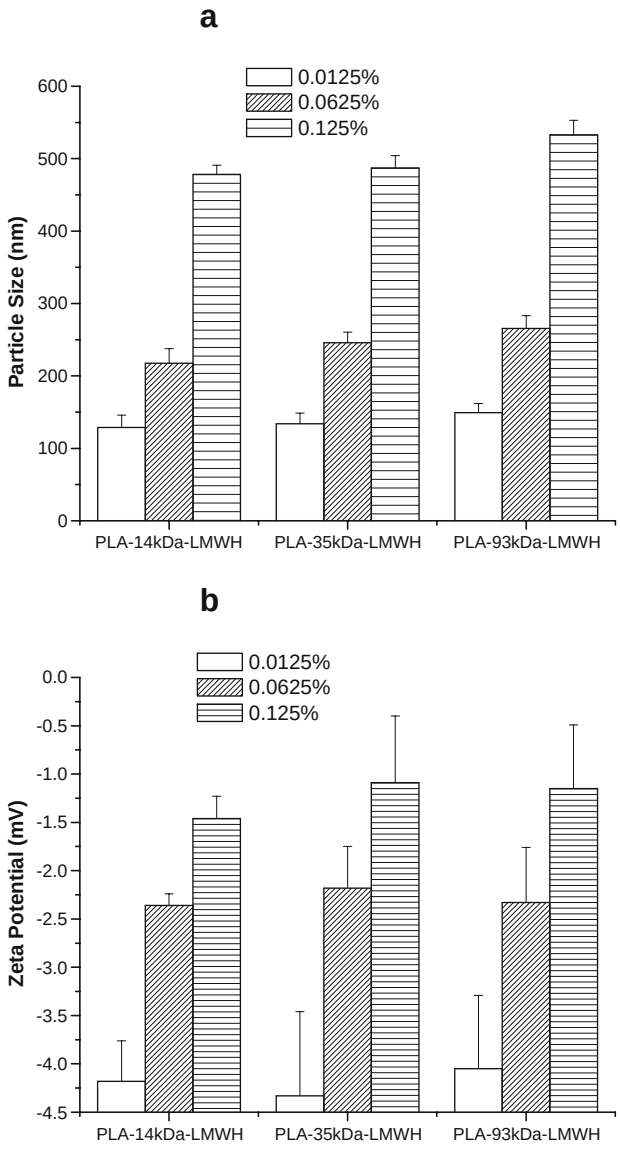

Fig. 1. Particle size analysis (**a**) and zeta potential (**b**) of enoxaparin–PLA complexes. Data represent mean±SD, $n=3$.

The zeta potential values for all complexes were negative, and the zeta potential became less negative with the increasing concentration of PLA (Fig. 1b). For example, the zeta potential for enoxaparin–PLA-14 kDa complexes decreased from -4.18 ± 0.41 to -1.4 ± 0.22 mV when the concentration of PLA was increased from 0.0125 to 0.125%. Similar to the increase in particle size, the magnitude of the zeta potential decreased in a concentration-dependent manner, but the molecular weight of the PLA did not affect the sign or magnitude of the zeta potential. The decrease in zeta potential agrees with the fact that addition of a positively charged polymer to a negatively charged drug will neutralize the negative surface charge of the drug. With the increasing concentration of the oppositely charged polymer, a gradual decrease in the net charge was observed; consequently, the zeta potential became less negative. This change in zeta

potential value upon addition of PLA agrees with previously published studies that showed that the surface charge of polyelectrolyte complexes depends on the drug-polymer ratios (27,28).

Quantitation of Enoxaparin–PLA Interactions

To test the hypothesis of electrostatic interactions between PLA and LMWH, the degree of complexation between PLA and enoxaparin was measured quantitatively by an azure A assay (29,30). Azure A is a positively charged dye which forms a purple colored complex with the negatively charged functional groups of enoxaparin. In enoxaparin–PLA complex, the azure A assay quantitatively measures unreacted (free) functional groups of LMWH and can be used as a measurement of free enoxaparin (31). No change in absorbance was observed when an increasing concentration of PLA was added to a solution containing a fixed amount of azure A (data not shown). However, for enoxaparin–PLA complexes, with increasing concentration of PLA a reduction in the amount of free LMWH was observed (Fig. 2). The reduction in the amount of free LMWH suggested that PLA interacts with negatively charged functional groups of LMWH. If no interaction between PLA and LMWH had occurred, there would have been no change in the amount of free LMWH in the presence PLA. These data also substantiate the data on particle size and zeta potential.

Transport of Mannitol across Calu-3 Cell Monolayers

Calu-3 cells have been widely used as an *in vitro* model for studies of pulmonary drug transport across bronchial epithelial cells, although these cells are biochemically and phenotypically different from mortal bronchial epithelial cells. Several research groups established the permeability characteristics and tight junction properties of Calu-3 cell lines and found them to be a good *in vitro* model of the bronchial epithelium (25, 32). The *in vitro*−*in vivo* correlation studies conducted by Mathia *et al.* (2002) showed that the Calu-3 human bronchial epithelial cell culture can reproducibly predict the absorption

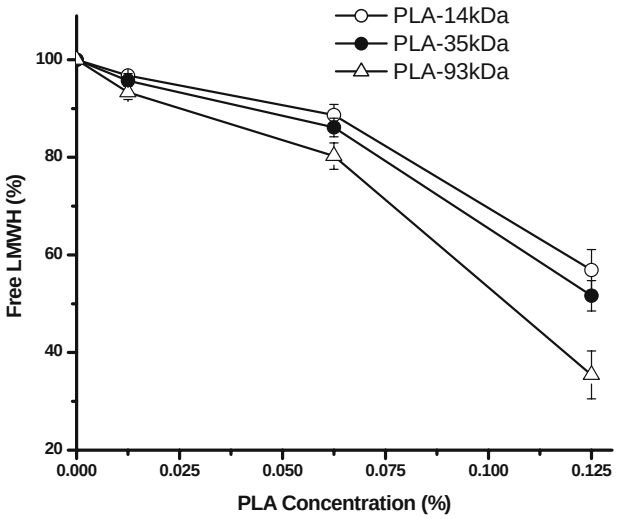

Fig. 2. Percentage of free enoxaparin in the different formulations upon complexation with PLA. Data represent mean±SD, n=3.

of drugs administered *via* the pulmonary route (33). To determine if poly-L-arginine (PLA) is able to open cell–cell tight junctions and increase the paracellular diffusion of a drug, the transport of ^{14}C-mannitol across Calu-3 cells was studied in the presence or absence of enoxaparin-PLA complex and over a range of PLA molecular weights. In the absence of enoxaparin-PLA complex, there was no transport of ^{14}C-mannitol across the cell monolayer from the apical chamber to the basolateral chamber during the course of the 2-h experiment (Fig. 3, normal saline control). However, when enoxaparin-PLA complex was present in the apical fluid, a molecular-weight-dependent and dose-dependent increase in ^{14}C-mannitol transport was observed (Fig. 3). Enoxaparin-PLA complex increased the movement of mannitol from the apical to the basolateral side of the monolayer even at the lowest concentration of PLA used, 0.0125%; the P_{app} was increased almost 20-fold (Fig. 3, Table I). Transport of ^{14}C-mannitol also increased as a function of the molecular weight of the PLA in the enoxaparin–PLA complex added to the apical side. The highest level of mannitol transport was observed with enoxaparin-0.125% PLA-93 kDa (p<0.05), and the P_{app} for this formulation was fourfold higher compared to that for 0.0125% PLA-93 kDa (Fig. 3c, Table I).

The increase in overall permeability of the monolayer to mannitol in the presence of enoxaparin–PLA complex suggests that PLA is able to open the tight junctions and enhance transport of mannitol *via* the paracellular route. This is consistent with previous reports (1,8). To confirm the hypothesis that PLA causes an opening of cell–cell tight junctions, the transport of mannitol was studied in the presence of varying concentrations of plain PLA-93 kDa. Previous data suggest that an increase in nasal absorption occurs with increasing molecular weight of a polycation (8). Thus, in this study only PLA-93 kDa was used, because its molecular weight is the highest of all PLAs tested.

A 0.125% concentration of PLA-93 kDa produced a profound increase in the P_{app} of mannitol transport (Table I); although lower concentrations of PLA-93 kDa (0.0625 and 0.0125%) also caused increases in P_{app}, the levels were less than 50% of that produced by 0.125% PLA-93 kDa (Table I). The observed reductions in P_{app} at the higher concentrations of PLA-93 kDa in the absence of its complexation with enoxaparin could be attributed to the preferential interaction of PLA with the negatively charged monolayer cells, rather than with mannitol, owing to the high charge density over the PLA molecules. From this study it can be concluded that absorption through the Calu-3 cell monolayer may be better explained on the basis of charge neutralization of the complexed molecule, in which it becomes more interactive with the cells, as opposed to the high positive charge of PLA alone.

In vitro TEER Determination across Calu-3 Cell Monolayers

Intact cell–cell tight junctions are a critical element of any cultured cell absorption model mimicking the *in vivo* conditions that govern drug transport (34). Reductions in TEER following the addition of an absorption enhancer have been used previously as an indicator of compromised epithelial cell–cell junctional stability inflicted by the absorption enhancer (35). TEER measurement can also be used to substantiate the transport of any solute across a cell mono-

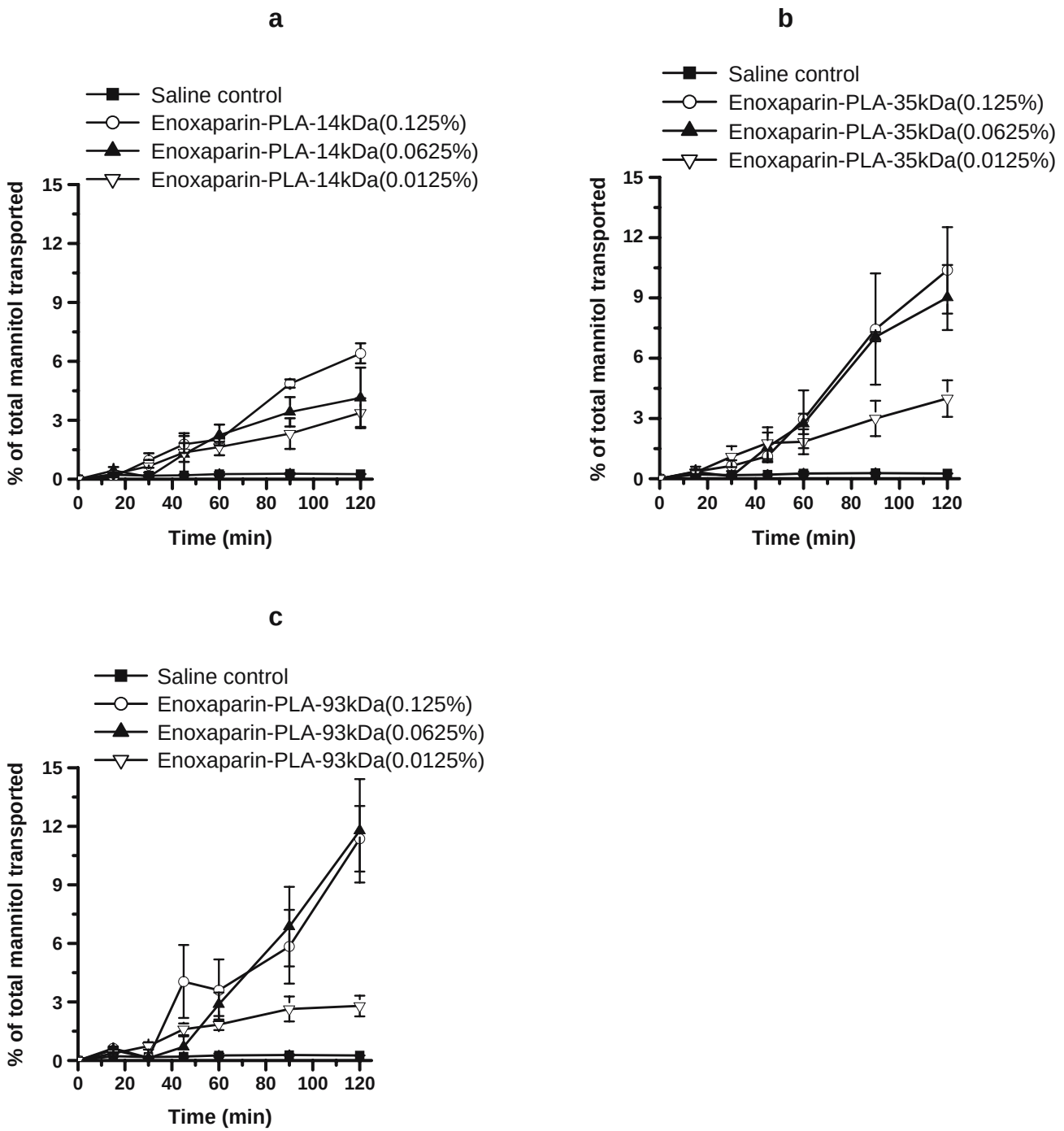

Fig. 3. Percentage of ^{14}C-mannitol transported across Calu-3 cell monolayers in the presence of various enoxaparin–PLA complexes with PLA-14 kDa (**a**), PLA-35 kDa (**b**) or PLA-93 kDa (**c**). Data represent mean±SD, n=3.

layer. Thus, in this study, TEER measurements across the Calu-3 cell monolayer in the presence or absence of enoxaparin–PLA complex were used to assess the effects of PLA on tight junction stability and the paracellular pathway. TEER across the cell monolayers was measured following a 120-min treatment with enoxaparin–PLA complex or normal saline in the apical chamber (Fig. 4). In the absence of any enhancer, there was no change in TEER during the entire measurement period. In contrast, addition of enoxaparin–PLA complex resulted in a gradual reduction in TEER values over 120 min, suggesting the opening of tight junctions and an increase in paracellular permeability. The highest concentration in the study, enoxaparin–PLA-93 kDa (0.125%), produced the greatest reduction in TEER, causing it to decrease to 55% of the initial value after 120 min. In the case of enoxaparin–PLA-93 kDa (0.0125%), the reduction in TEER was 84% of the initial value (Fig. 4c). Similar to the concentration-dependent decrease, the TEER values also decreased as the molecular weight of the PLA increased—the extent of the decrease in TEER can be ranked as PLA-93

Table I. Effects of PLA and Enoxaparin–PLA Complex on ^{14}C-mannitol Transport Across the Calu-3 Cell Monolayer

Formulations	Mannitol P_{app} $\times 10^{-7}$ (cm/s)
Plain enoxaparin in saline	1.4±0.3
Enoxaparin–PLA-14 kDa (0.0125%) complex	23.6±5.1
Enoxaparin–PLA-14 kDa (0.0625%) complex	28.9±10.7
Enoxaparin–PLA-14 kDa (0.125%) complex	44.7±3.6
Enoxaparin–PLA-35 kDa (0.0125%) complex	27.8±6.4
Enoxaparin–PLA-35 kDa (0.0625%) complex	62.8±11.3
Enoxaparin–PLA-35 kDa (0.125%) complex	72.3±15.0
Enoxaparin–PLA-93 kDa (0.0125%) complex	19.5±3.6
Enoxaparin–PLA-93 kDa (0.0625%) complex	82.1±18.5
Enoxaparin–PLA-93 kDa (0.125%) complex	79.2±11.7
PLA-93 kDa (0.0125%)	57.9±27.5
PLA-93 kDa (0.0625%)	63.9±9.5
PLA-93 kDa (0.125%)	141.5±46.5

Data Represent Mean ± SD (n=3).

kDa >PLA-35 kDa >PLA-14 kDa (Fig. 4). Overall, consistent with the results of the *in vitro* mannitol transport study above, the reductions in TEER values in the presence of enoxaparin–PLA complex were both dose-dependent and PLA-molecular-weight-dependent.

MTT Cytotoxicity Study

To evaluate the cytotoxicity of enoxaparin–PLA complex to Calu-3 cells, the viability of the cells was assessed with the MTT assay. MTT, a tetrazolium salt, is cleaved by mitochondrial dehydrogenase in living cells to form a measurable, dark blue product called formazan. Damaged or dead cells display a reduced dehydrogenase activity and diminished formazan production. As enoxaparin–PLA-93 kDa complex produced the greatest increase in cell permeability to mannitol and LMWH, this drug-polycation complex was used in the safety studies with MTT and in the BAL studies described below. Enoxaparin–PLA-93 kDa complexes containing a range of concentrations of PLA-93 kDa and sodium dodecyl sulfate (SDS) as a positive control were compared for their cytotoxic effects on Calu-3 cells following incubation for different time intervals. Results of the MTT test showed high levels of viability of Calu-3 cells after incubation with enoxaparin–PLA complex for up to 24 h; in contrast, only 15% of Calu-3 cells were viable following treatment with 0.1% SDS ($p<0.05$). Increasing the incubation time with enoxaparin–PLA complex did not elicit any significant Calu-3 cell toxicity, which is an important finding if PLA is to be used as a drug carrier (data not shown).

Pulmonary Absorption Studies in Rats

Studies of the pulmonary absorption of LMWH mediated by PLA as a carrier were performed by monitoring changes in plasma anti-factor Xa activity after intratracheal administration of enoxaparin–PLA complex. Initial studies were performed with plain enoxaparin to determine the optimal concentration at which the absorption-enhancing agent was able to produce a maximum increase in LMWH absorption and a therapeutic anti-factor Xa level in the rodent model. An anti-factor Xa level greater than 0.2 U/ml is considered to be therapeutic (36). When plain enoxaparin was administered to rats *via* the intratracheal route, a modest anti-factor Xa level was observed, although it was below the therapeutic level for an antithrombotic effect in male Sprague–Dawley rats (Fig. 5).

Administration of a pulmonary formulation containing enoxaparin plus different PLA combinations (i.e. PLA of three molecular weights at 0.0125, 0.0625 and 0.125% concentrations) increased the plasma anti-factor Xa activity to different extents (Fig. 5). When enoxaparin formulated with 0.0125% PLA-14 kDa was administered intratracheally, an appreciable increase in plasma anti-factor Xa level was observed compared to enoxaparin formulated in normal saline; however, it did not attain a therapeutic level in the blood (Fig. 5a). Similarly, enoxaparin plus 0.0625 or 0.125% PLA-14 kDa formulation failed to produce a therapeutic level of anti-factor Xa activity. However, enoxaparin plus 0.0125% PLA-35 kDa did produce a therapeutic anti-factor Xa level. When the concentration of PLA-35 kDa was increased to 0.0625%, the absorption of enoxaparin increased still more, although increase was not significant ($p>0.05$). When the concentration of PLA-35 kDa was further increased to 0.125%, there was an abrupt decrease in the anti-factor Xa activity as compared to 0.0625% PLA-35 kDa (Fig. 5b). A similar overall pattern was also found for the enoxaparin–PLA-93 kDa complexes. Enoxaparin plus 0.0125% PLA-93 kDa as well as 0.0625% PLA-93 kDa were able to produce therapeutic anti-factor Xa levels. However, when the concentration of PLA was increased to 0.125%, there was a significant decrease in the observed anti-factor Xa activity, regardless of the molecular weight of PLA used (Fig. 5c). This decrease in absorption with the increase in concentration of PLA can be explained by the particle size data obtained with the different complexes. The particle size of LMWH-0.125% PLA complex was in the range of 478–532 nm, where particles are more susceptible to phagocytosis due to their large size compared to the smaller particles (128–265 nm) produced by the 0.0125 or 0.0625% PLA concentration-based complexes (37). Based on the differences in the particle size of the different complexes, it can be hypothesized that larger particles are quickly cleared from the lungs because of phagocytosis, but smaller particles can traverse through the thin respiratory epithelium for absorption to occur. The data presented above also suggest that there was a molecular-weight-dependent effect of PLA on the pulmonary absorption of enoxaparin. For example, when the molecular weight of 0.0125% PLA was increased from 14 to 93 kDa, there was a significant increase of anti-factor Xa activity. Overall, the data presented in Fig. 5 demonstrate that PLA was efficacious as a carrier in enhancing pulmonary absorption of enoxaparin, and that such enoxaparin absorbed *via* the pulmonary route was able to increase anti-factor Xa levels.

The mechanism by which PLA enhances absorption of enoxaparin is not known. It was suggested that these cationic polymers could open the tight junctions of the membrane and

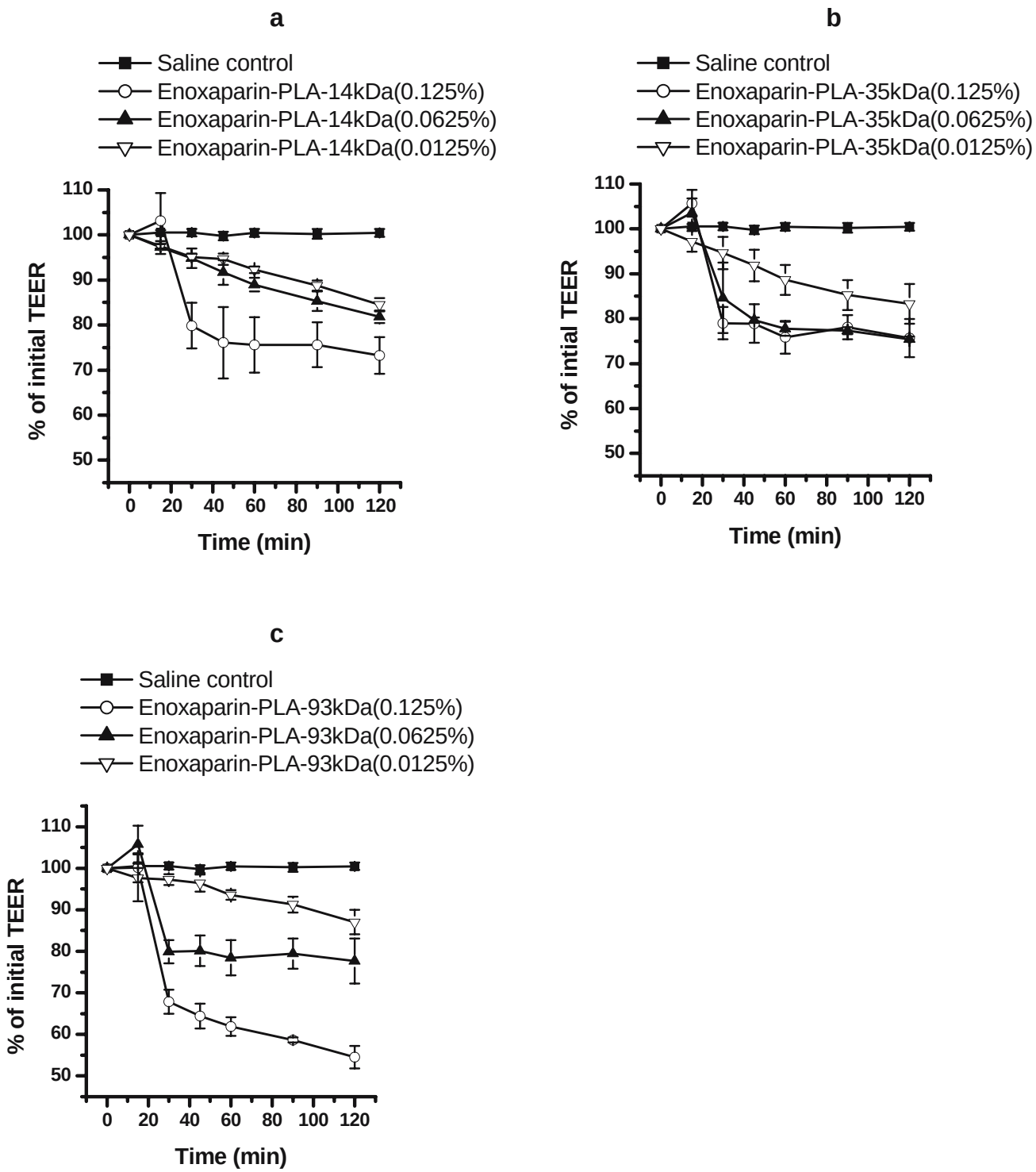

Fig. 4. Changes in TEER of Calu-3 cell monolayers in the presence of various enoxaparin–PLA complexes with PLA-14 kDa (**a**), PLA-35 kDa (**b**) or PLA-93 kDa (**c**). Data represent mean±SD, n=3.

increase the paracellular permeability of the epithelial membrane (8,38–40). The results of the *in vitro* mannitol transport and TEER studies described above also support such a mechanism.

Furthermore, as LMWHs are negatively charged drugs, cationic agents such as PLA are likely to neutralize the negative surface charges of LMWHs and thereby increase LMWH absorption *via* the pulmonary epithelium. McEwan *et al.* (41) reported that permeability changes induced by polycations were more dependent on the amount of positive charges than on the type of cationic moiety. In this study, when PLA concentration was increased from 0.0125 to 0.0625%, the pulmonary absorption of enoxaparin was also increased. The enhanced absorption may be because of the reduction of net negative charge of the drug due to an electrostatic interaction with PLA molecules. However, when PLA concentration was

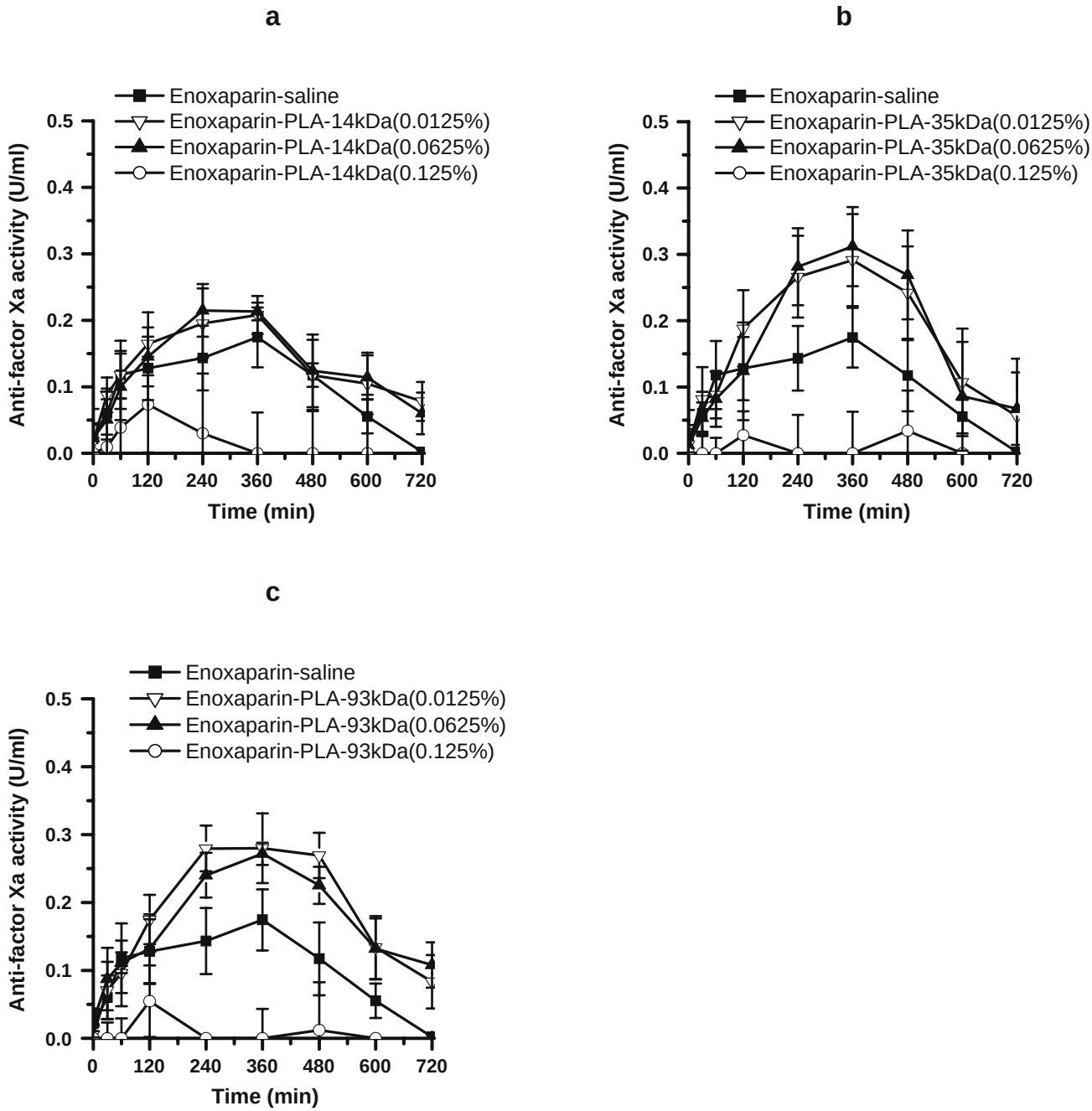

Fig. 5. Changes in plasma anti-factor Xa activity after pulmonary administration of enoxaparin (50 U/kg) in the absence or presence of PLA-14 kDa (**a**), PLA-35 kDa (**b**) or PLA-93 kDa (**c**). Data represent mean±SD, n=5–6.

increased to 0.125%, the absorption of enoxaparin decreased. As discussed above, the increased concentration of PLA perhaps causes excessive aggregation resulting in increased particle size to an extent which is more likely to be phagocytosed by macrophages of the lungs before absorption can occur.

In addition to the effects of net negative surface charges, the high molecular weight of PLA might also exert an important role on its absorption-enhancing effect. Schipper *et al.* (42) suggested that molecular weight played a very important role in determining the enhancing effects of chitosans, another positively charged biopolymer, on drug absorption. Indeed, larger molecular weight chitosans with higher degrees of deacetylation were able to increase the permeability of the epithelial membrane. Also, Natsume *et al.* (1) reported that L-arginine increased the absorption of FITC-dextran through the rat nasal mucosa as a function of PLA molecular weight. The bioavailabilities of FITC-dextran (4.4 kDa) following nasal administration were 24.7, 62.9 and 78.7% when PLAs with molecular weights of 8.9, 45.5 and 92.0 kDa, respectively, were used as absorption enhancers. Furthermore, Wang *et al.* (43) reported that the high molecular weight of aminated gelatin might have contributed to its absorption-enhancing effect and speculated that higher molecular weight enhancers themselves would present a low

level of penetration through the membrane, therefore demonstrating a stronger interaction with the mucosa and a stronger absorption-enhancing effect. In our view also, high molecular weight cationic permeation enhancers retained over the membrane for a prolonged time should elicit heightened membrane permeability.

Pharmacokinetic parameters of different enoxaparin formulations, presented in Table II, showed that the C_{max} values for formulations containing PLA-35 kDa and PLA-93 kDa were higher than those for formulations containing PLA-14 kDa. For example, C_{max} for the 0.0125% PLA-93 kDa formulation was approximately 1.3-fold higher than formulations containing PLA-14 kDa at the same concentration. As can be seen in Table II, with an increase in the concentration of PLA (from 0.0625% to 0.125%), there was a decrease in the C_{max} of the drug absorption profile. To assess the feasibility of pulmonary enoxaparin as an efficacious delivery system, relative bioavailability was calculated by comparing the bioavailability of intratracheally administered enoxaparin with that of subcutaneously administered enoxaparin. A dose- and molecular weight-dependent effect of PLA can be appreciated from the $AUC_{0\rightarrow720}$ and the bioavailability data presented in Table II. When the molecular weight of PLA was increased from 14 k to 93 kDa, the relative bioavailabilities were increased. The relative bioavailability data suggest that pulmonary delivery of enoxaparin can also be as effective as that of subcutaneous enoxaparin, and pulmonary delivery of LMWH could be a viable and non-invasive alternative to subcutaneous LMWH.

Bronchoalveolar Lavage Studies

Although polycationic molecules can work as absorption enhancers, their net positive charge leads to varying degrees of toxicity to the lung microenvironment (44). PLA is also known to cause damage to the airway epithelial cells in a concentration-dependent manner (45). As we have discussed earlier, PLA in this study was used to neutralize the negative surface charge of LMWH by forming an electrostatic complex. The zeta potential data also suggest that the surface of the complexes was negatively charged; therefore, high positive surface charge of PLA is likely to be reduced after its complexation with enoxaparin, and the resulting complex should not be as toxic as plain PLA. The bronchoalveolar lavage (BAL) study was performed in order to investigate if enoxaparin formulated with poly-L-arginine causes any biochemical or cellular changes in the lungs. Because toxicity often increases with the increase in molecular weight of polycationic molecules (11,46), in this study we chose to investigate the toxicity produced by the largest PLA molecule used at a maximum concentration of 0.125%. For the BAL studies, lipopolysaccharide (LPS) and SDS were used as positive controls. LPS is a complex lipopolysaccharide molecule present in the outer membrane of gram-negative bacteria and SDS is an anionic surfactant known for its harsh effect on the biological membrane. Both of these agents have been shown to cause lung damage and release lung injury markers such as lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and *N*-acetylglucosaminidase (NAG) (26,47). In this regard, the safety of poly-L-arginine based enoxaparin formulation was assessed and compared with LPS and SDS by monitoring the changes in the markers of lung injury in BAL fluid (Fig. 6). LPS produced a twofold increase in LDH levels 24 h after the treatment compared to normal saline and a 1.6-fold rise in LDH levels was observed in SDS-treated rats (Fig. 6a). No such increase in LDH levels was observed in animals treated with enoxaparin plus poly-L-arginine. Similar results were obtained when ALP levels were measured (Fig. 6b). For instance, LPS produced a 2.5-fold increase in ALP levels compared to normal saline and the ALP levels for the SDS-treated group were 1.6-fold greater than those for normal saline-treated group. In fact, the increase in ALP levels for the LPS-treated group correlated well with the increased LDH levels, although the increase in ALP levels was significantly less than for the LDH levels. However, the LDH and ALP levels for the enoxaparin plus poly-L-arginine-treated animals were the same as those observed in the rats treated with normal saline. The profiles of the NAG levels, presented in Fig. 6c, were also similar to the profiles of the LDH and ALP levels. Both SDS- and LPS-treated rats produced a 1.3- to 2-fold increase in NAG levels 24 h after the treatment. Furthermore, the differences between the NAG levels produced in the normal saline- and enoxaparin plus poly-L-arginine-treated rats were not statistically significant ($p>0.05$).

The biochemical changes that may occur in response to pulmonary exposure of a drug and pharmaceutical adjuvants have been studied by monitoring the changes in enzymatic

Table II. Pharmacokinetic Parameters for Intrapulmonary Delivery of Plain Enoxaparin (50 U/kg), Enoxaparin (50 U/kg) Formulated with Poly-L-Arginine, and Subcutaneous Plain Enoxaparin (50 U/kg)

Formulations	C_{max} (U/ml)	T_{max} (min)	$AUC_{0\rightarrow720}$ (U.min/ml)	$F_{relative}$ (%)
Plain enoxaparin in saline	0.18±0.04	400±48	53.5±16.8	41.4±10.3
0.0125% PLA-14 kDa	0.22±0.03	260±90	101.1±16.4*	62.1±10.1*
0.0625% PLA-14 kDa	0.23±0.03	300±69	103.1±18.4*	63.3±11.3*
0.125% PLA-14 kDa	0.06±0.03	600±120	13.2±4.5	8.1±2.8
0.0125% PLA-35 kDa	0.31±0.06*	264±70	124.5±30.6*	76.5±15.7*
0.0625% PLA-35 kDa	0.30±0.05*	320±62	123.7±29.4*	76.0±12.5*
0.125% PLA-35 kDa	0.08±0.02	480±85	24.5±6.3	15.0±3.8
0.0125% PLA-93 kDa	0.30±0.03*	300±65	134.3±35.2*	82.5±18.5*
0.0625% PLA-93 kDa	0.27±0.03*	312±66	124.0±15.4*	76.2±9.4*
0.125% PLA-93 kDa	0.12±0.04	600±90	23.9±7.6	14.7±4.7
Plain enoxaparin in saline subcutaneous	0.36±0.03	120±24	162.8±48.7	–

*Results are significantly different from those obtained without enhancer, $p<0.05$.
Data represent mean±SD (n=5–6).

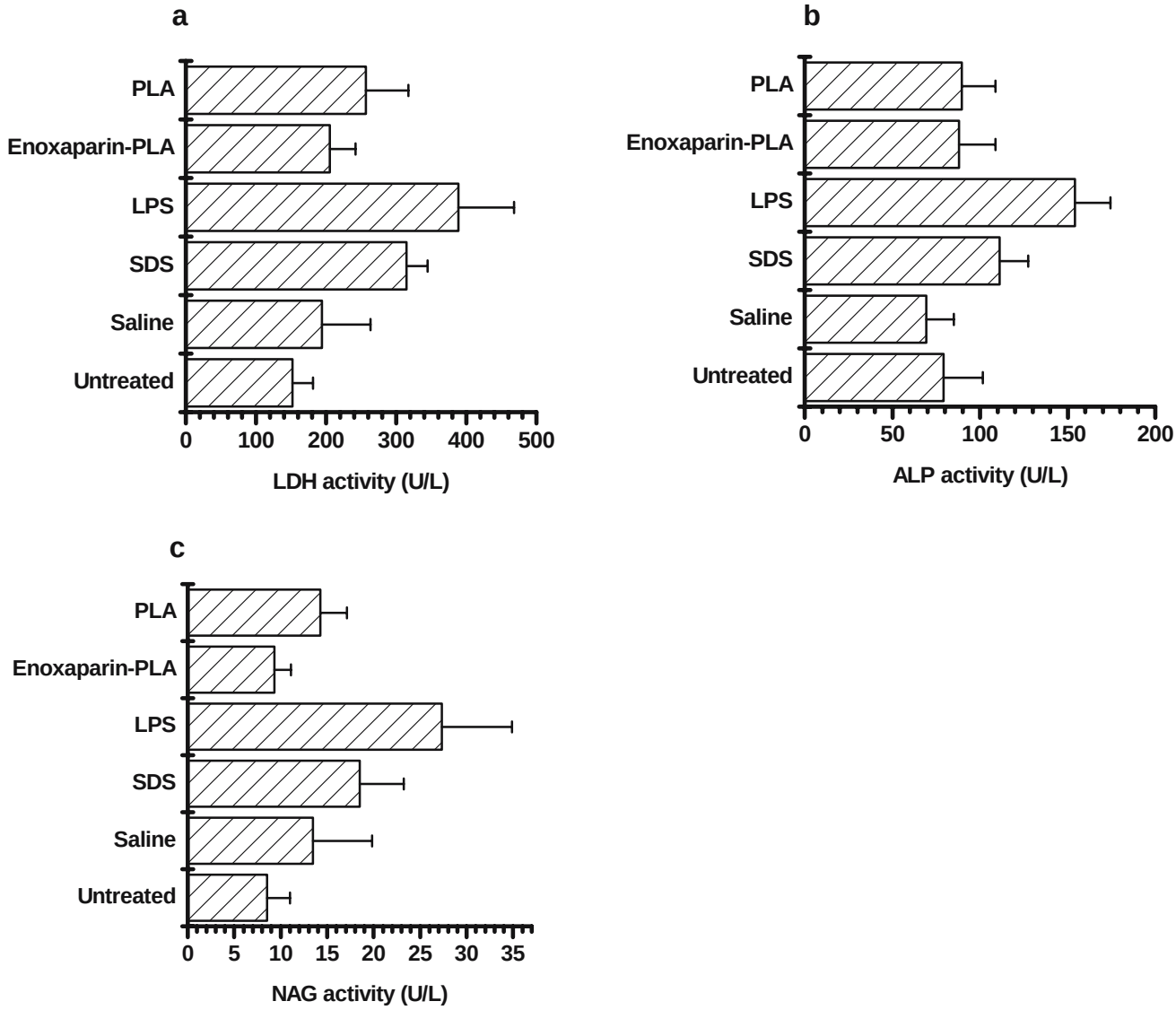

Fig. 6. Enzyme activities of LDH (**a**), ALP (**b**), and NAG (**c**) following bronchoalveolar lavage fluid analysis at 24 hours after pulmonary administration of enoxaparin-0.125% PLA-93 kDa complexes. Data represent mean±SD, n=5–6.

activities in bronchoalveolar lavage fluid (47). Of the enzymes studied, LDH, ALP and NAG are known to provide important insights as to the cell injury produced by exogenous substances. LDH, for example, is found in almost all body tissues, and under normal physiological states this enzyme remains localized within the cells. When cells are damaged or destroyed, the release of LDH into the blood stream or extracellular fluid causes its level to rise. Therefore, LDH is used as a general injury marker. Similarly, ALP, a membrane-bound indicator of type II cell secretory activity, is used as a sensitive marker of compromised cellular integrity or toxicity induced by cell damage or pathological conditions. It has been considered as an indicator of alveolar type II cell proliferation in response to type I cell damage (47,48). Elevated levels of lung-specific ALP were observed in the BAL fluid collected from Syrian hamsters exposed to oxidant gas, NO_2 (48). On the other hand, the presence of lysosomal enzymes such as NAG in the BAL fluid is used as an indication of increased phagocytic activity of cells in response to inhaled particles (49).

As discussed above, the LDH and ALP levels in SDS-treated rats imply that damage to lung cells was evident 24 h after the treatment. However, enoxaparin plus poly-L-arginine-treated rats showed enzymatic profiles similar to normal saline-treated rats. Altogether, the two positive controls used in the study produced significant damage to the lung tissue, whereas enoxaparin plus poly-L-arginine formulation did not cause any damage. These data are consistent with the hypothesis that poly-L-arginine is relatively safe for use in a pulmonary formulation. Overall, the BAL study demonstrates that poly-L-arginine is well tolerated by the respiratory epithelium.

Taken together, this study opens up the possibility of using polycationic poly-L-arginine as an efficacious carrier for the delivery of anionic moieties through the pulmonary route, if formulated judiciously. The approach described here, showing the use of poly-L-arginine carrier for enhanced delivery of low molecular weight heparin and with minimal cellular damage and lung injury, could be further optimized for different therapeutic molecules for *in vivo* use.

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