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Research Paper

Tumor-Targeted Gene Delivery Using Poly(Ethylene Glycol)-Modified Gelatin Nanoparticles: *In Vitro* and *in Vivo* Studies

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Purpose. To develop safe and effective systemically administered nonviral gene therapy vectors for solid tumors, DNA-containing poly(ethylene glycol)-modified (PEGylated) gelatin nanoparticles were fabricated and evaluated *in vitro* and *in vivo*.

Methods. Reporter plasmid DNA encoding for β-galactosidase (pCMV-β) was encapsulated in gelatin and PEGylated gelatin nanoparticles using a water-ethanol solvent displacement method under controlled pH and temperature. Lewis lung carcinoma (LLC) cells in culture were transfected with the pCMV-β in the control and nanoparticle formulations. Periodically, the expression of β-galactosidase in the cells was measured quantitatively using an enzymatic assay for the conversion of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) to *o*-nitrophenol (ONP). Qualitative expression of β-galactosidase in LLC cells was observed by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Additionally, the plasmid DNA-encapsulated gelatin and PEGylated gelatin nanoparticles were administered intravenously (i.v.) and intratumorally (i.t.) to LLC-bearing female C57BL/6J mice. At various time points postadministration, the animals were sacrificed and transgene expression in the tumor and liver was determined quantitatively by the ONPG to ONP enzymatic conversion assay and qualitatively by X-gal staining.

Results. Almost 100% of the pCMV-β was encapsulated in gelatin and PEGylated gelatin nanoparticles (mean diameter 200 nm) at 0.5% (w/w) concentration. PEGylated gelatin nanoparticles efficiently transfected the LLC cells and the β-galactosidase expression, as measured by the ONPG to ONP enzymatic conversion assay at 420 nm absorbance, increased starting from 12 h until 96 h post-transfection. The efficient expression of LLC cells was also evident by the X-gal staining method that shows blue color formation. The *in vivo* studies showed significant expression of β-galactosidase in the tumor following administration of DNA-containing PEGylated gelatin nanoparticles to LLC-bearing mice by both i.v. and i.t. routes. Following i.v. administration of pCMV-β in PEGylated gelatin nanoparticles, for instance, the absorbance at 420 nm per gram of tumor increased from 0.60 after 12 h to 0.85 after 96 h of transfection. After i.t. administration, the absorbance values increased from 0.90 after 12 h to almost 1.4 after 96 h.

Conclusions. The *in vitro* and *in vivo* results of this study clearly show that a long-circulating, biocompatible and biodegradable, DNA-encapsulating nanoparticulate system would be highly desirable for systemic delivery of genetic constructs to solid tumors.

KEY WORDS: Lewis lung carcinoma model; nonviral delivery; PEG-modified gelatin nanoparticles; plasmid DNA; tumor-targeting.

INTRODUCTION

The explosion of information obtained from the understanding at genetic, molecular, and cellular levels has opened new avenues in the prevention, diagnosis, and treatment approaches for cancer (1). Gene therapy represents a novel approach for treatment that is designed either to alleviate the genetic defect in tumor cells or to provide additional protective effect (2). Gene therapy strategies in solid tumors, using direct gene administration (*in vivo*) or removing cells for transformation (*ex vivo*), can be divided into methods that restore cellular growth control, confer drug sensitivity, induce antitumor immunity, and inhibit neo-angiogenesis (3,4).

For *in vivo* gene medicine to become a practical clinical reality in the 21st century, it is necessary to develop delivery vectors that will transport therapeutic genes to a specific region either locally or systemically in order to efficiently express encoded proteins at the target site (5). The vectors for gene delivery must have qualities of safety for repeated use and provide reproducible levels of the gene product. Based on the safety concerns with the viral vectors (6,7), there has been greater focus on the development of nonviral gene delivery vectors over the last few years (8,9). An ideal

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vector, in order to successfully transfect cells at a remote site, needs to be inert and stable while in circulation, yet once it reaches the target site should release the payload to effect an efficient and specific transfection of the target cells (10–12). Specific virus-like characteristics that must be included into nonviral vectors are small size and stability against aggregation in blood, serum or extracellular fluid, the ability to be efficiently internalized by the target cells, release of stable DNA in the cell once internalized, and allow the cytosolic-delivered DNA to be imported into the nucleus for transcription (13,14). Thorough understanding of the intracellular trafficking pathways and mechanisms to improve DNA stability are necessary pre-requisites for efficient gene delivery (15).

Nanoparticulate systems are attractive methods of DNA delivery because of their versatility, ease of preparation, and protection of the encapsulated plasmid DNA (16). These carrier systems can efficiently encapsulate various sizes of plasmids and provide protection during transit in the systemic circulation. They can also be targeted to reach specific tissues and cells in the body and avoid uptake by the mononuclear phagocytic system after systemic administration through the use of cell-specific ligands and attachment of poly(ethylene glycol) (PEG) chains on the nanoparticle surface (17–19). Nanoparticles usually have a high surface area to volume ratio and thus are able to efficiently encapsulate DNA even without pre-condensing step. Nanoparticles can also be made to reach a target site by virtue of their size and charge (20). For the delivery to solid tumors *in vivo*, long-circulating PEG-modified nanoparticles are preferentially distributed in the vasculature due to the *enhanced permeability and retention* (EPR) effect (21–23). Jain's group has shown that the effective pore size of most peripheral human tumors range from 200 nm to 600 nm in diameter, with a mean of about 400 nm (24,25). Additionally, liposomes and nanoparticles with a positive surface charge are taken preferentially in the tumor and retained for longer duration as compared to negatively charged or neutral particles (26).

For the development of polymeric nanoparticulate carriers with improved encapsulation and efficient intracellular delivery of DNA, we have outlined the following design criteria (27). First, the matrix polymer must be biocompatible, and preferably biodegradable. Second, the carrier system should efficiently encapsulate the DNA, retain its supercoiled structure, and protect it during transit in the systemic circulation. Third, the carrier should be able to reach specific tissues and cells in the body and avoid uptake by the mononuclear phagocytic system after systemic administration. Fourth, the nanoparticulate system should be able to deliver the DNA inside the cell through nonspecific or receptor-mediated endocytosis and provide protection to the payload during cellular transit and nuclear import. Finally, for industrial production, the system should be amenable to scale-up and manufacturing under the Current Good Manufacturing Practices guidelines.

Based on these design criteria, we have developed and evaluated PEGylated gelatin nanoparticles as a noncondensing plasmid DNA carrier system for systemic delivery to solid tumors (28). Gelatin is a proteinaceous biopolymer, traditionally obtained by acidic or basic hydrolysis of collagen (29). It has a long history of safe use in pharmaceuticals, cosmetics, as well as, food products and it is considered as a "generally regarded as safe (GRAS)" material by the United States Food and Drug Administration (30). Injectable form of gelatin that is sterile and pyrogen-free is commercially available. In addition, due to the potential hazards of animal-derived materials, FibroGen (South San Francisco, CA, USA) has developed gelatin by recombinant DNA technology (http://www.fibrogen.com).

We have synthesized PEG-epoxide, modified Type-B gelatin with PEG-epoxide to form PEGylated gelatin, and reproducibly prepared nanoparticles with various payloads using a mild water-ethanol solvent displacement method under controlled pH and temperature conditions (31,32). When PEGylated gelatin nanoparticles were encapsulated with plasmid DNA encoding for enhanced green fluorescent protein (EGFP-N1), we found high efficient transfection efficiency in NIH-3T3 murine fibroblast cells after 96 h post-transfection (33). Recently, we have examined the biodistribution profile and tumor targeting potential of radiolabeled PEGylated gelatin nanoparticles in Lewis lung carcinoma-bearing female C7BL/6J mice (34). PEGylated gelatin nanoparticles were shown to preferentially target the tumor mass and approximately 4–5% of the intravenously injected dose remained in the tumor for up to 12 h postadministration. In the current study, we have encapsulated reporter plasmid DNA encoding for β-galactosidase (pCMV-β) in the control and PEGylated gelatin nanoparticles and examined quantitatively and qualitatively the transfection potential *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Type-B gelatin (225 bloom strength) with 100–115 mmol of free carboxylic acid per 100 g of protein, an isoelectric point of 4.7–5.2, and an average molecular weight 40,000–50,000 daltons was purchased from Sigma Chemical Company (St Louis, MO, USA). Monomethoxy-poly(ethylene glycol) (PEG) with a molecular weight of 5,000 daltons was obtained from Fluka Chemika/Biochemika (Ronkonkoma, NY, USA). Reporter plasmid DNA (1.0 mg/ml in endotoxin-free deionized distilled water), expressing the enzyme β-galactosidase (pCMV-β), was purified by Elim Biopharmaceuticals, Inc. (Hayward, CA, USA). Lipofectin, a cationic lipid transfection reagent, was purchased from Invitrogen (Carlsbad, CA, USA). General-purpose serum free medium (SFM, UltraCulture) was obtained from Bio-Whittaker, (Walkersville, MD, USA). The *o*-nitrophenyl-β-D-galactopyranoside (ONPG) based kit for quantitative β-galactosidase expression analysis in both cell lysates and tissue homogenates and the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining kit for cells and tissue microscopy studies were purchased from Stratagene (La Jolla, CA, USA). The OCT (optimum cutting compound) tissue mounting medium was obtained from Tissue-Tek; Miles Scientific (Elkhart, IN, USA). Reagent grade dehydrated ethanol was purchased from Fisher Scientific (Milwaukee, WI, USA). All aqueous solutions were prepared exclusively in deionized distilled water (Nanopure II, Barnstead/Thermolyne, Dubuque, IA, USA).

Preparation and Characterization of Plasmid DNA-Encapsulated Nanoparticles

Nanoparticles of the unmodified gelatin and PEGylated gelatin derivative (with 30% of the available amine groups modified using 0.5 g PEG-epoxide to 1.0 g of gelatin) containing plasmid DNA expressing β-galactosidase were prepared by the ethanol precipitation method under controlled conditions of temperature and pH as previously described (31,32). Briefly, 200 mg of gelatin or PEGylated gelatin derivative was dissolved in 20 ml of deionized distilled water at 37°C until a clear solution was obtained. The pH of the resulting solution was adjusted to 7.00 with 0.2 M sodium hydroxide. The plasmid DNA was available in 1.0 mg/ml concentration solution in endotoxin-free deionized distilled water. The DNA solution was added to the gelatin or PEGylated gelatin solution such that the final DNA concentration of 0.5% (w/w). The nanoparticles were formed by gradual addition of the aqueous biopolymer solution in dehydrated ethanol under controlled stirring conditions. In the final mixture of 100 ml, the ethanol to water volume ratio was maintained constant at 65:35. Crosslinking of the nanoparticles was carried out by the addition of 1.0 ml of 40% (w/v) glyoxal and stirring for another 10 minutes. Any unreacted aldehyde groups of glyoxal were quenched with aqueous 12% (w/v) sodium metabisulfite and the plasmid DNA-containing nanoparticles were centrifuged at 14,000 rpm for 90 minutes in a Ti70 rotor fitted onto a Beckmann LE 80 K centrifuge, washed twice with deionized distilled water, and lyophilized.

Prior to lyophilization, the resulting turbid gelatin or PEGylated gelatin nanoparticle suspension was analyzed for mean particle size and size distribution with a Coulter counter. A sample of the diluted (1:4) nanoparticulate suspension in deionized distilled water was used for particle size analysis at a scattering angle of 90° and a temperature of 25°C using Beckman/Coulter N4 plus (Fullerton, CA, USA) instrument. The capacity and efficiency of β-galactosidase plasmid DNA loading in the gelatin and PEGylated nanoparticles was determined by the protease digestion method (33). A sample (50 mg) of the DNA-containing nanoparticles was incubated with 0.2 mg/ml protease in phosphate buffered saline (PBS, pH 7.4) at 37°C for 30 min until a clear solution was obtained. The released DNA was quantified from the emission intensity measurements of PicoGreen-complexed DNA.

In Vitro Transfection Studies in Lewis Lung Carcinoma Cells

Cell Culture Conditions

Lewis Lung carcinoma (LLC) cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in culture in Dubelco's modified Eagle medium (DMEM) supplemented with glucose, L-glutamine, HEPES buffer, Pen-strep, and fetal bovine serum at 37°C and 5% CO_2. LLC was selected as a model cell line for *in vitro* transfection studies in order to optimize the conditions of *in vivo* transfection in LLC-bearing mice. The tumor cell line was reported by ATCC to be free of murine pathogens (bacteria or viruses).

Quantitative and Qualitative Transgene Expression

Quantitative Transgene Expression. In order to quantify the transgene expression in the cytosol of LLC cells exposed to pCMV-β in the control and nanoparticle formulations, an enzymatic assay was developed. The activity of β-galactosidase in the cell lysates was measured by the conversion of a colorless ONPG solution into yellow-colored o-nitrophenol (ONP) product as described by Nielsen *et al.* (35). Approximately 10,000 LLC cells per well were grown in DMEM overnight in 6-well microplates. A suspension of plasmid DNA-containing gelatin or PEGylated nanoparticles was prepared at a concentration of 2.5% (w/v) polymer concentration in SFM. The media was replaced with plasmid DNA-containing gelatin or PEGylated nanoparticles and plasmid DNA-Lipofectin complexes. Each well received the equivalent of 20 μg of plasmid DNA from all of three formulations. Control samples of LLC cells did not receive any plasmid DNA. After 6 h of incubation to allow the DNA-containing nanoparticles and Lipofectin systems to enter the cells, the SFM with excess carriers was removed and the plates were washed with 2.0 ml of sterile phosphate buffered saline (PBS, pH 7.4) three times and after which, the media was replaced with DMEM.

Periodically, following transfection with pCVM-β, the cells were treated with trypsin-EDTA, centrifuged, and the supernatant in the wells was discarded. The cell pellet was reconstituted in 200 μl of the Stratagene kit LYSIS buffer. The lysed pellet was centrifuged at 1,000 rpm for 10 minutes to remove cell debris. The supernatant (50 μl) was pipetted into a 96 well plate and 110 μl of Stratagene kit Buffer A with mercaptoethanol was added, followed by incubation at 37°C for 5 minutes. The substrate ONPG (50 μl) was added to each well and the plates were kept at 37°C for an additional 2.5 h. The enzymatic conversion of ONPG to ONP was stopped by the addition of 20 μl of Stratagene kit STOP solution. The visible absorbance of the formed ONP was measured at 420 nm using a microplate reader and the values were normalized to 10,000 cells.

Qualitative Transgene Expression. After initial passage in tissue culture flasks, LLC cells were grown to semi-confluence in supplemented DMEM in 6-well tissue culture plates on Corning's circular glass clean cover-slips at 37°C and 5% CO_2 atmosphere. A suspension of plasmid DNA-containing gelatin or PEGylated nanoparticles was prepared at a concentration of 2.5% (w/v) polymer con-centration in SFM. DNA-Lipofectin complexes were pre- pared by mixing plasmid DNA with Lipofectin in SFM. Each well received equivalent of 20 μg of plasmid DNA from the polymeric nanoparticle suspension or Lipofectin-complexed DNA. The cells were incubated with the DNA carriers for 6 h to allow for internalization in the cells. SFM with excess carriers was removed and the plates were washed three times with 2.0 ml sterile PBS and the media was replaced with DMEM.

At specified time intervals post-transfection, the cells were fixed for 1.0 h with 2.0 ml of fixing solution containing 7.0% (v/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in sterile PBS. The fixatives were washed twice with sterile PBS and the cells were incubated for 30 minutes at 37°C with X-gal staining solution containing 100 μl of X-gal, and 20 μl each of 400 mM potassium ferricyanide, 400 mM potassium ferrocyanide, and 200 mM magnesium chloride

solutions in PBS. The individual cover-slips were mounted cell side up on clean glass slides with 70% glycerol. Bright-field images were acquired with a Zeiss Axioplan-2 microscope (Thornwood, NY, USA) at an original magnification of 40×. The images were digitized and processed with Adobe Photoshop software.

In Vivo Transfection Studies in Lewis Lung Carninoma Tumor Model

Tumor Model Development

The *in vivo* transfection efficiency of β-gal plasmid administered in the gelatin or PEGylated gelatin nanoparticle formulation was evaluated in the LLC solid tumor model (36). All the animal experiments described here were approved by the Northeastern University's Institutional Animal Care and Use Committee. Female C57BL/6J mice (6–8 weeks, 25 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). The animals were provided food and water *ad libitum* and housed in an environment of controlled temperature and humidity with 12-h cycles of light and darkness. Prior to any experimentation, the animals were allowed to acclimate in their new habitat for at least 48 h.

We have selected LLC as a solid tumor model since it is a relatively faster growing murine tumor and does not cause secondary metastasis. Also, our previous experience showed that LLC model allowed for reproducible induction of tumors in female C57BL/6J mice (37). Finally, Weissig *et al.* (38) has shown that the effective pore size of the blood vessels in LLC-bearing mice is significantly smaller than other murine tumor models. As such, LLC-bearing mice model provided a challenge for passively-targeted DNA delivery based on the EPR effect using PEGylated gelatin nanoparticles.

To initiate subcutaneous tumors, the left hind flank region of each mouse was shaved and disinfected. Approximately, 30,000 LLC cells suspended in 100–200 μl in sterile Hank's balanced salt solution were injected subcutaneously into the shaved left hind flank region of ether-anesthetized mice. Following tumor inoculation, the animals were monitored daily until subcutaneous tumors were palpable and approximately 10–15 mm in diameter. During the time of tumor development and subsequent administration of the control and test formulations, the health of animals was monitored regularly by measuring their body weight.

In Vivo Administration of Plasmid DNA in the Nanoparticle Formulations

Plasmid DNA (CMV-β)-containing gelatin and PEGylated gelatin nanoparticles were suspended in sterile 0.9% (w/v) sodium chloride solution by vortexing in order to get a stable suspension with de-aggregated nanoparticles. After induction of light anesthesia, each mouse received an amount equivalent to 20 μg dose of plasmid DNA either intravenously (i.v.) via the tail vein or intratumorally (i.t.) by directly injecting into the center of the tumor mass with a 25-gauge needle. At specified time points of 12, 24, 48, and 96 h postadministration, the animals were sacrificed by CO_2 inhalation. The biodistribution studies of gelatin and PEGylated gelatin nanoparticles in LLC-bearing C57BL/6J mice reported earlier showed that the predominant distribution of these nanoparticles occurred in the tumor and liver only (34). As such, following sacrifice, the tumor and liver tissues were excised and weighed immediately. The excised organs were washed in 2.0 mM magnesium chloride containing PBS on ice to get rid of any excess blood and other debris. The tissues were then fixed for approximately 8 h in 4% (v/v) paraformaldehyde in PBS at 4°C. They were then washed twice in 2.0 mM magnesium chloride containing PBS to get rid of any fixative as the residual fixative can potentially inhibit the activity of β-galactosidase enzyme. Tumor and liver samples were then immersed in 30% (w/v) sucrose solution at 4°C for 12 h to protect during freezing. The tissues were then washed and immersed in ice-cold isopentane for about 3.0 min. The frozen tissues were stored at −80°C until further use.

Quantitative and Qualitative Transgene Expression

Quantitative Transgene Expression

The expression of β-galactosidase in tumor and liver homogenates was quantitated by measuring the enzymatic activity upon conversion of clear ONPG to yellow-colored ONP. After thawing, the tumor and liver samples were homogenized in 10 ml of ice-cold PBS and treated with 2.0 mg/ml collagenase to degrade the connective tissue. The tissue homogenates were treated for 5.0 minutes in 0.4% (v/v) paraformaldehyde in PBS, centrifuged, washed three times with PBS, and then reacted with 0.5 ml of 5 mM ONPG in pH 7.7 sodium phosphate buffer. Quantitative expression of β-galactosidase in the tumor and liver samples was analyzed by measuring the absorbance at 420 nm using a Shimadzu UV/VIS spectrophotometer (Columbia, MD,

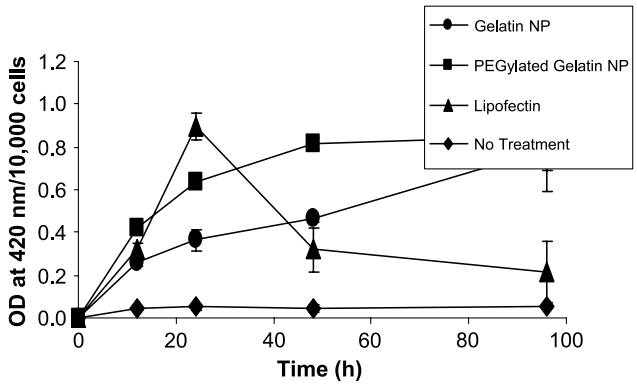

Fig. 1. *In vitro* quantitative transgene expression measured in Lewis lung carcinoma cells after incubation with plasmid DNA encoding for β-galactosidase (pCMV-β) that was encapsulated in the gelatin and poly(ethylene glycol)-modified (PEGylated) gelatin nanoparticle formulations. DNA-complexed Lipofectin, a cationic lipid transfection reagent, was used as a control. The transfection efficiency in the cell lysates as a function of time was quantitated by an enzymatic assay using visible absorbance at 420 nm upon conversion of the colorless *o*-nitrophenyl-β-D-galactopyranoside solution into yellow-colored o-nitrophenol. Results are expressed as absorbance or optical density (OD) at 420 nm normalized per 10,000 cells (mean ± S.D., $n = 4$).

USA). The reported absorbance values were normalized to the weight of the tumor or liver samples in grams.

Qualitative Transgene Expression

To develop cryogenic sections of the excised tumor and liver samples, they were sliced and mounted in OCT and 8 μm-thick slices were cut with a microtome. After mounting the samples on clean glass slides, the tissues were stained overnight in PBS containing 0.4 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside), 2 mM magnesium chloride, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide. The tissue sections were then fixed with 60% (w/v) glycerol in water and the expression of β-galactosidase was observed from the blue staining. Brightfield images were acquired with a Zeiss Axioplan-2 microscope. The images were digitized and processed with Adobe Photoshop software.

Data Analysis

Analysis of the experimental results was performed using a student *t* test with Microsoft Excel (Redmond, WA, USA) software on Windows NT computer platform. Statistical significance was evaluated at 95% confidence interval ($p < 0.05$). Mean and standard deviation of the results are reported.

RESULTS AND DISCUSSION

Properties of Plasmid DNA-Containing Nanoparticles

Based on the isoelectric point of Type B gelatin (4.7–5.2), a higher pH of 7.0 was intentionally selected to prepare the gelatin and PEGylated gelatin nanoparticles in order to insure that the DNA would be entrapped, rather than electrostatically complexed, in the polymer matrix. The Coulter particle analysis data showed that the encapsulation method using water-ethanol solvent displacement under controlled temperature and pH could reproducibly prepare DNA-containing gelatin and PEGylated gelatin nanoparticles of approximately 100 to 500 nm in diameter with an average particle size of 200 nm. Almost 100% of the CMV-β plasmid DNA was encapsulated in the gelatin or PEGylated gelatin nanoparticles at 0.5% (w/w) concentration.

Our previous studies confirmed that the mild formulation process used to encapsulate plasmid DNA in the gelatin and PEGylated gelatin nanoparticles maintains supercoiled

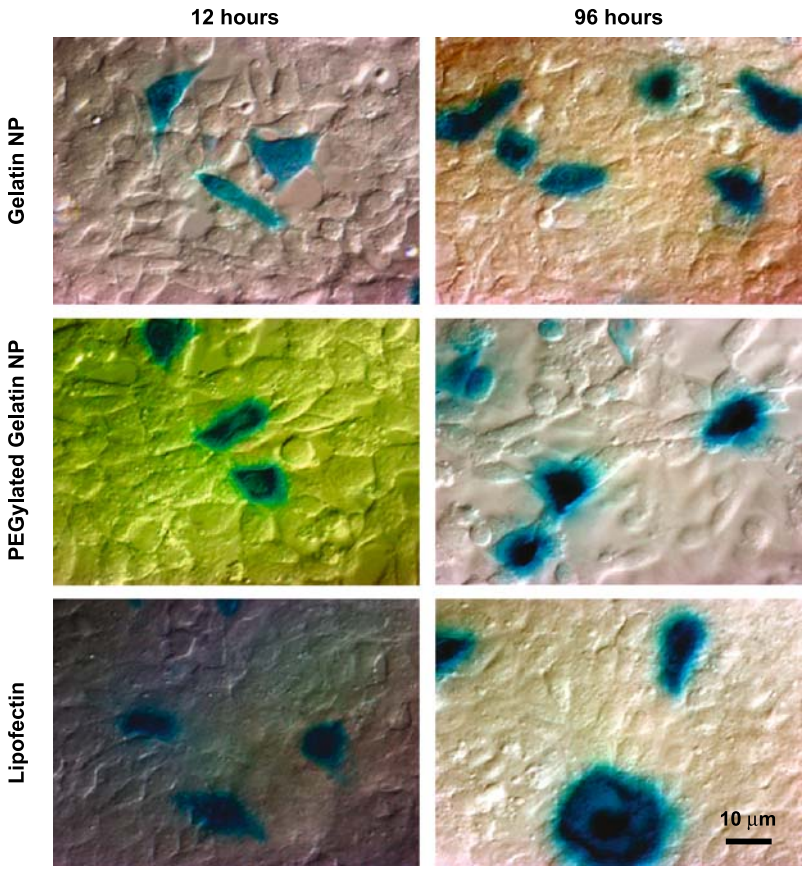

Fig. 2. Brightfield microscopy images of Lewis lung carcinoma cells in culture transfected with plasmid DNA encoding for β-galactosidase that was encapsulated in the gelatin and poly(ethylene glycol)-modified (PEGylated) gelatin nanoparticle formulations. DNA-complexed Lipofectin, a cationic lipid transfection reagent, was used as a control. Transgene expression after 12 and 96 h was qualitatively evaluated by staining the cells with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) that results in the blue color formation.

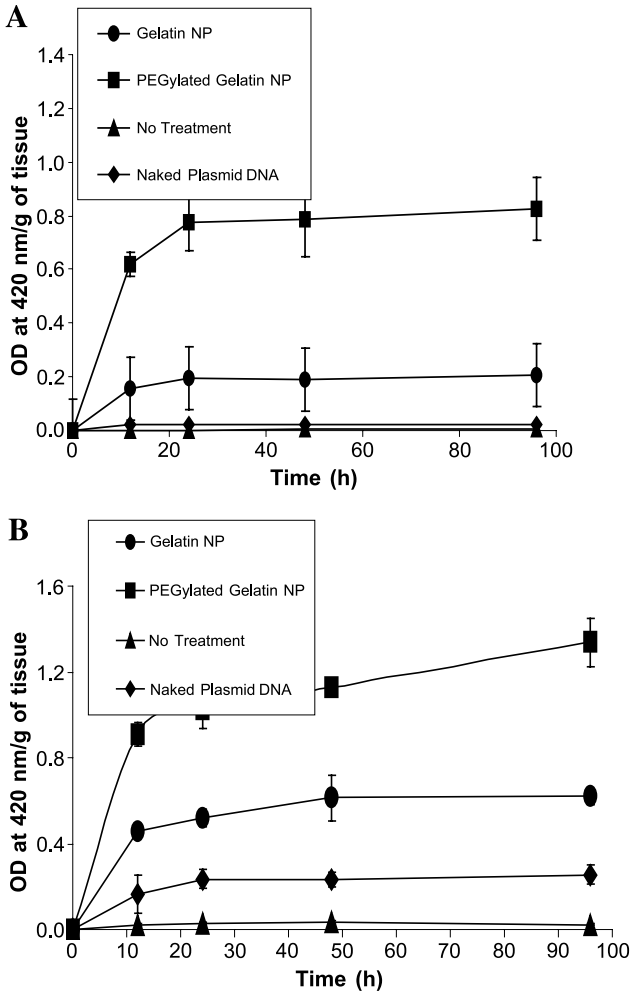

Fig. 3. *In vivo* quantitative transgene expression measured in the tumor mass of Lewis lung carcinoma-bearing female C57BL/6J mice administered with plasmid DNA encoding for β-galactosidase (pCMV-β) that was encapsulated in the gelatin and poly(ethylene glycol)-modified (PEGylated) gelatin nanoparticle formulations. The control (naked plasmid DNA) and nanoparticle formulations were administered by intravenous (A) and intratumoral (B) routes. The transfection efficiency in the tumor homogenates as a function of time was quantitated using visible absorbance at 420 nm by an enzymatic assay upon conversion of the colorless *o*-nitrophenyl-β-D-galactopyranoside solution into yellow-colored o-nitrophenol. Results are expressed as absorbance or optical density (OD) at 420 nm normalized per gram of tumor mass (mean ± S.D., $n = 4$).

structure of the plasmid DNA, as was shown by agarose gel electrophoresis (33). The gel electrophoresis results also showed that when the DNA-containing gelatin or PEGylated gelatin matrix was treated with DNAse-I, which was then subsequently quenched, and then treated with protease, the resulting released DNA did not undergo degradation as shown by the lack of fragmentation bands in the gel. However, when proteolytic degradation by protease preceded the DNAse-I treatment, there was extensive fragmentation as the released DNA was now accessible by DNAse-I. Overall, these results suggested that the plasmid DNA was completely encapsulated (as opposed to complexed or surface adsorbed) in the nanoparticle formulations, and hence would remain stable in the presence of serum and cytosolic degrading enzyme (33).

Furthermore, we postulate that the intracellular delivery of supercoiled plasmid DNA at the nuclear membrane is necessary for efficient transfection. Even if the DNA delivery system can be targeted to specific cells and necessary approaches are taken to prevent DNA degradation during intracellular trafficking, the poor transfection efficiency of cationic lipids and polymers is mainly due to the structural transition of uncoupled DNA from supercoiled to open-circular form at the peri-nuclear region (39). The open-circular plasmid DNA molecule, with a hydrodynamic diameter of >100 nm is just too large to enter through the nuclear membrane, which typically has a pore size ranging from 9 to 26 nm (40). In a noncomplexing matrix, such as PEGylated gelatin nanoparticles, the electrostatic uncoupling is not necessary, and as such, there is a higher probability that the released plasmid DNA at the peri-nuclear region will retain its supercoiled structure.

In Vitro Transfection Studies in Lewis Lung Carcinoma Cells

Quantitative Transgene Expression

Figure 1 shows the absorbance values at 420 nm corresponding to the formation of ONP from ONPG by the transfected β-galactosidase enzyme in the cell lysate. Previously, we have shown approximately 43% and 61% transfection efficiencies after 96 h in NIH-3T3 mouse fibroblast cells when green fluorescence protein encoding plasmid DNA was delivered in gelatin and PEGylated gelatin nanoparticles, respectively (33). The absorbance values at 420 nm increased consistently as a function of time with both gelatin and PEGylated gelatin nanoparticle-mediated DNA delivery. The maximum absorbance of approximately 0.80 was seen after 48 h with PEGylated gelatin nanoparticles. In contrast, after 48 h, the absorbance value for gelatin nanoparticle-mediated transfection was 0.42. Lipofectin-complexed plasmid DNA was able to transfect efficiently after 24 h with an absorbance peaking at 0.90. However, with increasing duration the β-galactosidase expression decreased as shown by reduction in the absorbance levels. Although, in the current study, we cannot directly measure the *in vitro* transfection efficiency of DNA delivered with the nanoparticle formulations, the absorbance *versus* time profile in Fig. 1 is very similar to that observed in our previous study with enhanced green fluorescent protein transfection in NIH-3T3 cells (33). Lipofectin-pCMV-β complexes-mediated transfection results also mirror those obtained earlier with Lipofectin- DNA complexes encoding for enhanced green fluorescent protein (33).

Qualitative Transgene Expression

The results of X-gal stained LLC cells transfected with pCVM-β using gelatin and PEGylated gelatin nanoparticles and Lipofectin-complexed DNA is shown in Fig. 2. As can be seen from the micrographs, the LLC cells were transfected and the expression of β-galactosidase was evident from 12 h and remained stable for up to 96 h postadministration of the plasmid DNA. For Lipofectin-complexed DNA, we

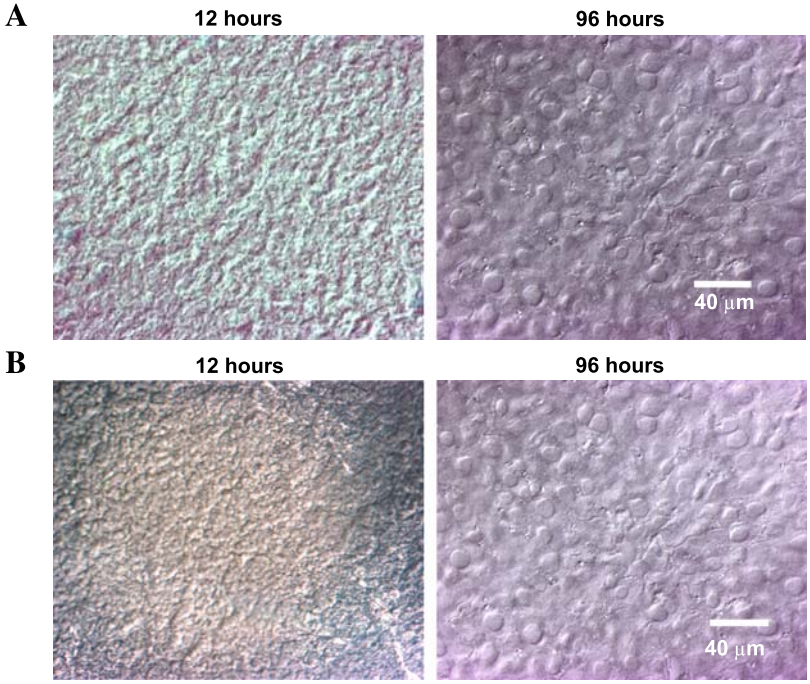

Fig. 4. Brightfield microscopy images of tumor cryogenic sections from Lewis lung carcinoma-bearing female C57BL/6J mice administered with naked plasmid DNA encoding for β-galactosidase (pCMV-β) intravenously (A) and intratumorally (B) after 12 and 96 h post-transfection. Transgene expression was qualitatively evaluated by staining the tumor sections with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) that results in the blue color formation.

observed that a significantly higher fraction of the cell population in the wells had succumbed to the cytotoxic effect of the cationic lipid reagent with increasing duration of incubation. The lower transgene expression efficiency, as shown by decreasing absorbance in Fig. 1, may be due to the cytotoxic effect of this reagent. Gelatin and PEGylated gelatin nanoparticles, on the other hand, were found to be completely nontoxic in cell viability assays (31).

In Vivo Transfection Studies in Lewis Lung Carcinoma Model

Transgene Expression in Tumor Mass

The absorbance at 420 nm due to the transfected β-galactosidase-catalyzed formation of ONP from ONPG in tumor mass as a function of time following i.v. and i.t. administration of the DNA in gelatin and PEGylated gelatin nanoparticles is shown in Fig. 3. The *in vivo* transfection capabilities of Lipofectin- and polyethyleneimine-complexed plasmid DNA in tumor mass after i.v. administration in LLC-bearing mice were also examined. However, neither of these reagents produced β-galactosidase levels, as measured by the absorbance values, that were any different from the untreated control (data not shown).

The results of i.v. and i.t. administration of pCMV-β in PEGylated gelatin nanoparticles clearly show that these systems were significantly superior in transfecting tumor mass. Following i.v. administration, for instance, the absorbance at 420 nm per gram of tissue increased from 0.60 after 12 h to 0.85 after 96 h of transfection. After i.t. administration, the absorbance values increased from 0.90 after 12 h to almost 1.4 after 96 h. Assuming that the i.t. administration provides the upper limit in terms of transfection efficiency, based on these absorbance values, the i.v. administration of DNA-containing PEGylated gelatin nanoparticle would be 61% as efficacious as i.t. However, it is important to note that not all of the DNA delivered in the PEGylated gelatin nanoparticles i.t. are expected to transfect. Considering the transport issues after systemic administration and the uptake by reticuloendothelial systems, it is remarkable that such a high transfection efficiency was observed with i.v. administration of the DNA-containing PEGylated gelatin nanoparticles. Plasmid DNA delivered in gelatin nanoparticles, on the other hand, resulted in significantly lower absorbance values at 420 nm after i.v. and i.t. administration. After 12 h for instance, the absorbance values were 0.16 and 0.41 for the i.v. and i.t. routes of administration, respectively. Increasing the duration to 96 h, increased the absorbance from DNA-delivered in gelatin nanoparticles to 0.18 and 0.62 for the i.v. and i.t. routes, respectively. For gelatin nanoparticles, the i.v. route of administration resulted in 29% transfection relative to the i.t. route.

From the biodistribution studies reported earlier (34), we have found that the residence half-life in the tumor of PEGylated gelatin nanoparticles (~121 h) was significantly higher as compared to the gelatin nanoparticles (~19 h). The longer residence time of the PEGylated gelatin nanoparticles in the tumor mass provide greater opportunity for uptake into the cells. Additionally, the higher stability of encapsulated supercoiled plasmid DNA in the cytosol and efficient uptake into the nucleus probably led to the higher transfec-

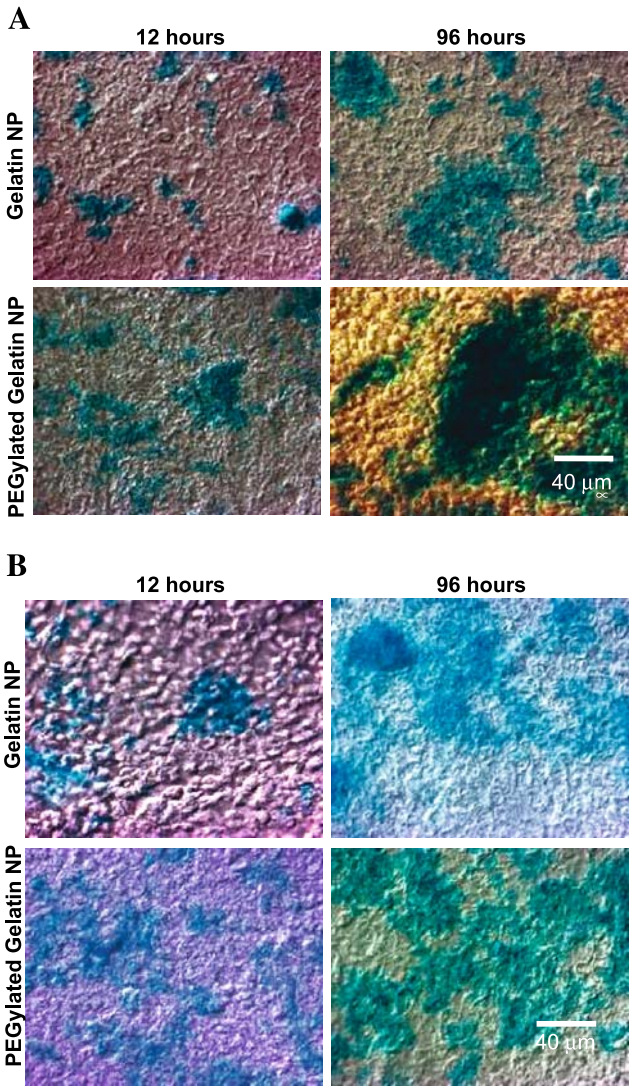

Fig. 5. Brightfield microscopy images of tumor cryogenic sections from Lewis lung carcinoma-bearing female C57BL/6J mice administered with plasmid DNA encoding for β-galactosidase (pCMV-β) in the gelatin and poly(ethylene glycol)-modified (PEGylated) gelatin nanoparticle formulations. The DNA-containing nanoparticle suspension was injected intravenously (A) and intratumorally (B) and the tumor sections were obtained after 12 and 96 h post-transfection. Transgene expression was qualitatively evaluated by staining the tumor sections with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) that results in the blue color formation.

tion efficiency observed with PEGylated gelatin nanoparticles in the tumor *in vivo*. With a significantly lower effective pore-size of the vasculature for passive targeting through the EPR effect in LLC-bearing mice (36), it is important to note that this model was specifically selected to optimize the conditions for efficient *in vivo* transfection.

Figure 4 shows the microscopic images of 8-μm-thick tumor cryogenic sections stained with X-gal for the presence of transfected β-galactosidase after administration of the naked plasmid DNA by the i.v. and i.t. routes. As expected, the naked plasmid was probably degraded very rapidly in serum and in the cytosol and, therefore, there was no evidence of transfection in any of these tissue sections. On the other hand, in Fig. 5, β-galactosidase expression is clearly observed in the tumor cryogenic sections after i.v. and i.t. administration of the plasmid DNA in gelatin and PEGylated gelatin nanoparticles. Significant number of blue patches, indicative of β-galatosidase transfection, was present after both the i.v. and i.t. administered tumor section upon administration of the plasmid in PEGylated gelatin nanoparticles after 12 and 96 h. The results obtained from qualitative analysis of tumor sections cannot be directly correlated with the quantitative results of ONPG to ONP conversion by expressed β-galactosidase. Although more than ten tumor sections were stained with X-gal and representative tissue sections are shown in Fig. 5, the sections still represent a very small portion of the entire tumor mass that was used for quantitative analysis.

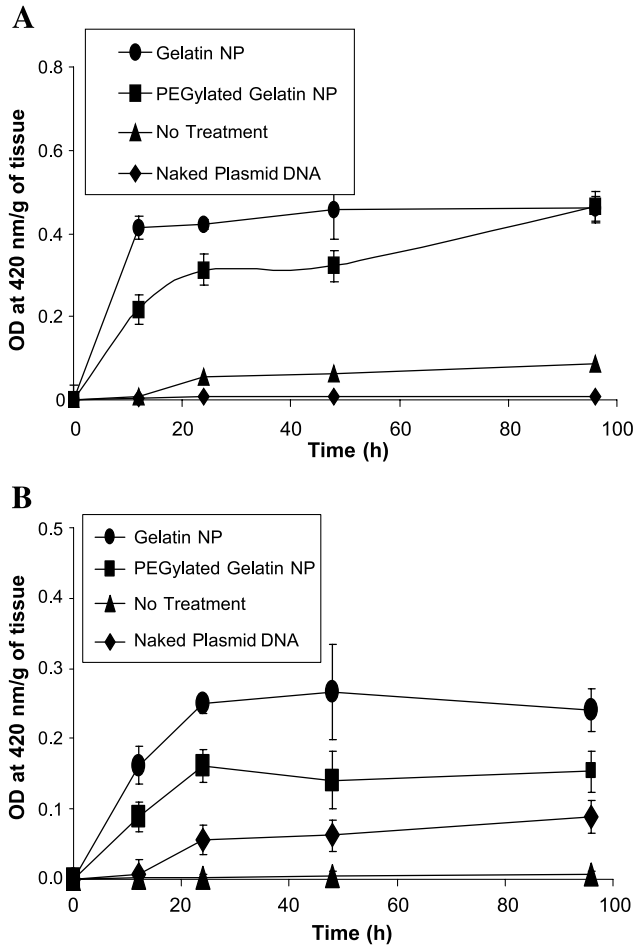

Fig. 6. *In vivo* quantitative transgene expression measured in the liver of Lewis lung carcinoma-bearing female C57BL/6J mice administered with plasmid DNA encoding for β-galactosidase (pCMV-β) that was encapsulated in the gelatin and poly(ethylene glycol)-modified (PEGylated) gelatin nanoparticle formulations. The control (naked plasmid DNA) and nanoparticle formulations were administered by intravenous (A) and intratumoral (B) routes. The transfection efficiency in the liver homogenates as a function of time was quantitated using visible absorbance at 420 nm by an enzymatic assay upon conversion of the colorless o-nitrophenyl-β-D-galactopyranoside solution into yellow-colored *o*-nitrophenol. Results are expressed as absorbance or optical density (OD) at 420 nm normalized per gram of liver tissue (mean ± S.D., $n = 4$).

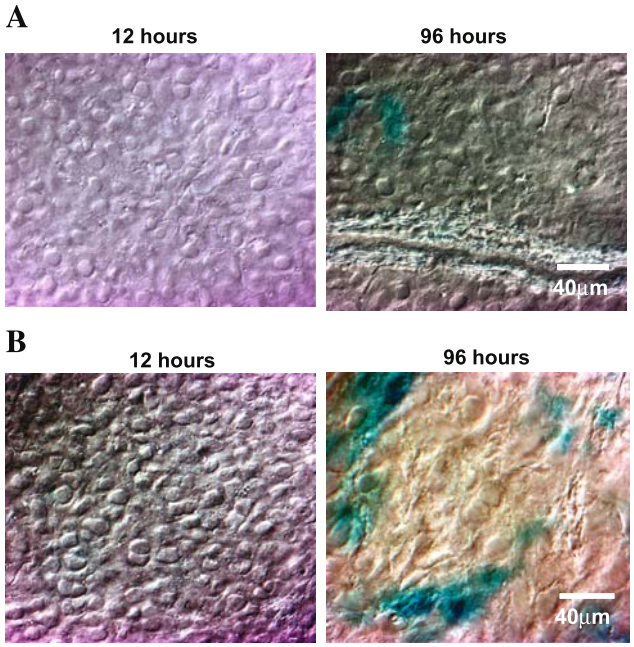

Fig. 7. Brightfield microscopy images of liver cryogenic sections from Lewis lung carcinoma-bearing female C57BL/6J mice administered with naked plasmid DNA encoding for β-galactosidase (pCMV-β) intravenously (A) and intratumorally (B) after 12 and 96 h post-transfection. Transgene expression was qualitatively evaluated by staining the liver sections with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) that results in the blue color formation.

Transgene Expression in the Liver

Being a nonspecific carrier of DNA, gelatin and PEGylated gelatin nanoparticles were also distributed significantly in the liver following systemic administration in LLC-bearing mice (34). After 2 h following i.v. administration, nearly 80% of the recovered dose of administered gelatin nanoparticle was found in the liver. In contrast, only 18% of the recovered dose of PEGylated gelatin nanoparticles was present in the liver after 2 h. The lesser uptake of PEGylated gelatin nanoparticles by the liver was due to the long-circulating property in the nanoparticles imparted by surface-accessible PEG chains, which prevents complement activation and macrophage-induced opsonization by the reticuloendothelial system (liver and spleen) following systemic administration (34).

Following i.v. and i.t. administration of β-galactosidase expressing plasmid DNA encapsulated in the gelatin and PEGylated gelatin nanoparticles, the transgene expression in the liver was determined quantitatively using the ONPG to ONP conversion assay. Figure 6 shows the absorbance values at 420 nm due to the formation of ONP in β-galactosidase transfected liver homogenates as a function of time. After i.v. administration, gelatin nanoparticles concentrate in the liver to a higher degree than the PEGylated gelatin nanoparticles. The absorbance at 420 nm after 12 h, for instance, was 0.42 and 0.22 for plasmid DNA delivered in gelatin and PEGylated gelatin nanoparticle formulations, respectively. However, after 96 h, the absorbance values from the liver ho- mogenates following i.v. administration for both forms of the nanoparticles reached the same plateau value of about 0.42. Interestingly, the results of i.t. administration also showed that there was higher β-galactosidase expression in the liver with gelatin nanoparticles as compared to PEGylated gelatin nanoparticles. For instance, after 12 h of transfection, the absorbance values at 420 nm were 0.17 and 0.08 for gelatin and PEGylated gelatin nanoparticles, respectively. These results would suggest that the DNA-containing gelatin nanoparticles were able to enter the systemic circulation following i.t. administration and reach the liver at higher concentrations that the PEGylated gelatin nanoparticles.

Figure 7 shows the microscopic images liver cryogenic sections stained with X-gal for the presence of transfected β-

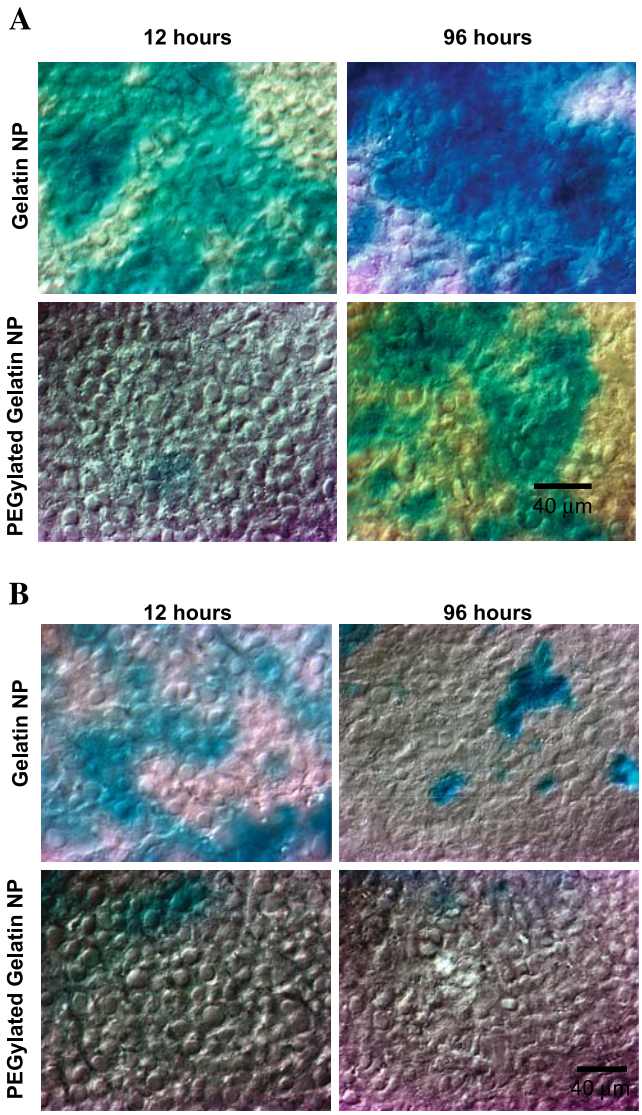

Fig. 8. Brightfield microscopy images of liver cryogenic sections from Lewis lung carcinoma-bearing female C57BL/6J mice administered with plasmid DNA encoding for β-galactosidase (pCMV-β) in the gelatin and poly(ethylene glycol)-modified (PEGylated) gelatin nanoparticle formulations. The DNA-containing nanoparticle suspension was injected intravenously (A) and intratumorally (B) and the liver sections were obtained after 12 and 96 h post-transfection. Transgene expression was qualitatively evaluated by staining the liver sections with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) that results in the blue color formation.

galactosidase after i.v. and i.t. administration of the naked plasmid DNA. In contrast to the tumor samples shown in Fig. 4, there were a few blue patches observed following both i.v. and i.t. administration of the DNA after 96 h of transfection. In addition, in Fig. 8, β-galactosidase expression is clearly observed in the liver cryogenic sections after i.v. and i.t. administration of the plasmid DNA in gelatin and PEGylated gelatin nanoparticles. We observed significantly greater number of blue patches in the liver section with DNA delivered in the gelatin nanoparticles as opposed to those with DNA delivered in PEGylated gelatin nanoparticles. These results corroborate the quantitative studies of β-galactosidase-mediated ONPG to ONP conversion discussed above in Fig. 6.

CONCLUSIONS

Development of safe and efficient systemically-administered nonviral gene delivery system is a major challenge that needs to be overcome in order for gene medicine to become a clinical reality in the 21st century. In this study, we have formulated plasmid DNA (pCMV-β)-containing gelatin and PEGylated gelatin nanoparticles (average diameter 200 nm) for targeted systemic delivery to solid tumors. Quantitative and qualitative β-galactosidase transfection results in LLC cells showed that PEGylated gelatin nanoparticles were superior transfection reagents as compared to gelatin nanoparticles and Lipofectin. More importantly, the results of *in vivo* expression of β-galactosidase in tumor mass showed that PEGylated gelatin nanoparticles could transfect with 61% efficiency after i.v. administration relative to i.t. administration. The *in vitro* and *in vivo* transfection efficiencies of PEGylated gelatin nanoparticles was attributed to a biocompatible, biodegradable, long-circulating carrier system that does not complex with DNA. A noncomplexing matrix would preserve the supercoiled structure of the plasmid DNA at the peri-nuclear region, which is critically important for nuclear import and efficient transfection.

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