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Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery☆

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Abstract

Calcium phosphate nanoparticles present a unique class of non-viral vectors, which can serve as efficient and alternative DNA carriers for targeted delivery of genes. In this study we report the design and synthesis of ultra-low size, highly monodispersed DNA doped calcium phosphate nanoparticles of size around 80 nm in diameter. The DNA encapsulated inside the nanoparticle is protected from the external DNase environment and could be used safely to transfer the encapsulated DNA under in vitro and in vivo conditions. Moreover, the surface of these nanoparticles could be suitably modified by adsorbing a highly adhesive polymer like polyacrylic acid followed by conjugating the carboxylic groups of the polymer with a ligand such as *p*-amino-1-thio-β-galactopyranoside using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride as a coupling agent. We have demonstrated in our studies that these surface modified calcium phosphate nanoparticles can be used in vivo to target genes specifically to the liver.

Keywords: DNA; Calcium phosphate; Non-viral vector

1. Introduction

In recent years with the advent of recombinant DNA technology, a surge in research activity has occurred in the field of DNA transfer across cell lines. This activity, which has taken the shape of what is popularly known as gene therapy, is a medical/surgical intervention technique which is being developed as a 'molecular medicine' and requires genes to be introduced into cells in order to treat a wide variety of till now incurable human diseases.

The field has evolved over the past few decades, with most gene therapy studies based on the use of viruses to deliver the gene (Anderson, 1998). Although viral vectors are attractive in terms of the scientific strategy of exploiting natural mechanism, such systems suffer from inherent difficulties of effective pharmaceutical processing, immunogenicity, scale up and the possibility of reversion of an engineered virus to the wild type (Davis, 1997; Verma and Somia, 1997). Consequently, a major focus is now being given to the

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development and use of non-viral vectors for safe and efficient DNA delivery (Luo and Saltzmann, 2000; Liu and Huang, 2002; Meyer and Finer, 2001).

A number of non-viral vectors have been explored till now. Some of these include lipid based carriers (Maurer et al., 1999), polycationic lipids (Cullis and Chonn, 1998), polylysine (Zauner et al., 1998), polyornithine (Ramsay et al., 2000), histones and other chromosomal proteins (Schwartz, 1999), hydrogel polymers (Leong, 1998; Perez et al., 2001) and precipitated calcium phosphate (CaPi) (Graham and van der Eb, 1973). Among these, the technique of calcium phosphate co-precipitation for in vitro transfection is used as a routine laboratory procedure (James and Grosveld, 1987). This method relies heavily on the fact that divalent metal cations, such as Ca^{2+}, Mg^{2+}, Mn^{2+} and Ba^{2+} can form ionic complexes with the helical phosphates of DNA (Truong-Le et al., 1999). Calcium phosphate, therefore, forms complexes with the nucleic acid backbone and thus may impart a stabilizing function to certain DNA structures. The complexes can then be carried across cell membrane via ion channel mediated endocytosis (Truong-Le et al., 1999). Orrantia and Chang (1990) estimated the intracellular distribution of DNA internalized through calcium phosphate precipitation and speculated that the low transfection with CaPi–DNA complex may be due to two reasons viz. (i) most of the endocytosed DNA is quickly degraded and excreted to the cytosol and (ii) small fraction of the remaining exogenous DNA macromolecules important for gene transfer may be delivered directly by intermediary vesicles from the endosomal compartment to the nucleus without traversing the cytosol.

As a matter of fact, although calcium phosphate precipitation method is simple, effective and still widely used in laboratory for in vitro transfection, the method is hampered by the difficulty of applying it to in vivo studies, especially delivery of DNA to any particular cell type. Due to bulk precipitation of calcium phosphate, the method also suffers from variation in calcium phosphate–DNA particle size that causes variation among experiments (Luo and Saltzmann, 2000).

We, therefore, suggest that these two wrongfully conceived myths regarding the use of calcium phosphate mediated gene delivery, viz., (i) low transfection efficiency and (ii) inability to apply in vivo condition, can be overcome by preparing ultra-low size calcium phosphate nanoparticles entrapping DNA molecules. It is envisaged that if these two impediments are overcome, calcium phosphate mediated gene delivery can become more advantageous compared to other viral and non-viral carriers in the sense that the method is relatively safe (since this is in the GRAS list of FDA), as well as cost effective. With the prospect of reviving this methodology of using calcium phosphate as carriers by improving on the preparative conditions, we have tried to devise a strategy that would make the process more efficient and useful, as well as suitable for in vivo applications. In this paper we have reported the transgene expression of β-galactosidase using pSVβgal as reporter gene (Zhang et al., 2001), The enzyme activity detected was over and above that of background endogenous activity.

2. Materials and methods

2.1. Materials

Surfactant Aerosol OT, of analytical grade, was obtained as a 96% pure product from ACROS ORGANICS (Belgium). *N*-hexane, of 99.5% purity, was purchased from SRL (India). Analytical grade agarose, calcium chloride and disodium hydrogen phosphate were obtained from SRL (India). pUC19 DNA (400 μg/ml) was a product of Bangalore Genei (India). Mammalian cell-line (Jurkat) and pSVβgal DNA were a kind donation from Dr Balaram Ghosh, Center for Biochemical Technology, Delhi. Gel-loading dye (bromophenol blue) and ethidium bromide were purchased from SIGMA-ALDRICH (USA). All other chemicals used were of the highest grade commercially available.

2.2. Preparation of calcium phosphate nanoparticles

Sodium bis(ethylhexyl)sulphosuccinate (AOT) of 0.1 M in hexane solution was prepared. In 25 ml of AOT in hexane, 50 μl of aqueous solution of $CaCl_2$ (20% w/v), 400 μl double distilled water and 10 μl of pDNA (400 μg/ml) were dissolved by continuous stirring for 72 h to form microemulsion A. In another 25 ml of AOT in hexane, 50 μl of aqueous solution of Na_2HPO_4 (5% w/v), 350 μl of double distilled water, 50 μl of 0.2 M Tris–HCl buffer (pH 6) and 10 μl of pDNA (400 μg/ml) were dissolved by continuous stirring for 48 h to form microemulsion B. Both the microemulsions were optically clear solutions. Then, microemulsion B was slowly added to microemulsion A at the rate of 5 ml/h with continuous stirring at 8–10 °C. The resulting solution was then, further stirred for another 6 h after which it was centrifuged at 8×10^3 rpm for half an hour. The pelleted nanoparticles were washed with hexane three times and finally it was redispersed in 10 ml of double distilled water by sonication (frequency 22 kHz; power 120 W) for 2 h. The dispersed nanoparticles were dialysed for 10 h and was kept at −4 °C for further use.

2.3. Preparation of galactopyranoside-tagged calcium phosphate nanoparticles encapsulating pSVβGal plasmid DNA

Galactose moiety was tagged to the particle surface using the following procedure. Ten microliter of aqueous dispersion of CaPi–DNA nanoparticles were incubated with 100 μl of 0.63% w/v polyacrylic acid (PAA) (mol.wt. 6.5 kDa) for 12 h, followed by dialysis (12 kDa cut off membrane) to remove excess polymer. PAA was conjugated to *p*-amino-1-thio-β-D-galactopyranoside (PAG) using (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride) (EDCI). Ten microliter of PAG (20 mg/ml) in Tris–HCl buffer (pH 7.0) was added to nanoparticle suspension with continuous stirring and to this 10 μl of EDCI (0.2 mg/ml) was added. Stirring was allowed to continue for 1 h, followed by prolonged dialysis for 10 h.

2.4. Determination of size of the nanoparticles by dynamic light scattering

Two microliter of pre-lyophilized aqueous dispersion of nanoparticles was used for laser light scattering experiment. The measurements were done with a Brookhaven BI 8000 instrument fitted with a BI200SM goniometer. An Argon-ion air-cooled laser was operated at 488 nm as a light source and the measurements were done on a scattering angle of 90°. The time dependent auto-correlation function was derived using a 136-channel digital photon correlator. The particle size was automatically calculated from the auto-correlation function using Stokes–Einstein equation.

2.5. Transmission electron micrograph

One drop of the pre-lyophilized sample solution (containing dispersed nanoparticles) was put on a formvar coated copper grid (1% solution of formvar was prepared in spectroscopic grade chloroform) and air-dried in a desiccator. The dried grid was then examined under an electron microscope (JEOL JEM2000 Ex200 model) at a magnification of 40 k.

2.6. Determination of encapsulating efficiency (E%)

The reverse micelle solution, after preparation of DNA loaded nanoparticles, was centrifuged at 8×10^3 rpm for 30 min when the encapsulated DNA in calcium phosphate was pelleted while the un-entrapped DNA remained in the aqueous core of reverse micelles. The *E*%, which refers to the amount of plasmid encapsulated in CaPi nanoparticles compared to the amount of plasmid added during encapsulation process in the reverse micelles, was determined by measuring the amount of free plasmid that was not encapsulated and therefore, remained in the supernatant upon centrifugation of the nanoparticle suspension. The amount of plasmid was determined spectrophotometrically at 260 nm against blank AOT/hexane solution as reference. The concentration of free DNA in the supernatant liquid after the

separation of the loaded CaPi nanoparticles by centrifugation was found to be undetectable spectrophotometrically. The hexane, therefore, was completely removed in an evaporator and the glassy residue was redissolved in 3 ml hexane. The OD was measured at 260 nm and was found to be very small. The $E\%$ was calculated from $[\{(P_o - P_f)/P_o\} \times 100]$ where P_o and P_f are the concentrations of plasmid originally added and present in the supernatant reverse micellar solution, respectively. The DNA concentration was determined from the standard plot of known DNA concentration versus OD at 260 nm (data not shown).

2.7. Agarose (1%) gel electrophoresis of free, entrapped and adsorbed pUC19 DNA

Twenty microliter of aqueous dispersion of CaPi–DNA nanoparticles (containing 0.07 μg/μl of pUC19 DNA) prepared by the above method were incubated with 5 μl of DNaseI (5 mg/ml in tris buffer, pH 5.5) for 30 min at room temperature (at which temperature the enzyme can completely degrade the DNA) and then loaded in a 1% agarose gel. As a control same amount of untreated nanoparticles containing the equal amount of pUC19 DNA was loaded. In other lanes 2.0 μl of free pUC19 DNA (400 μg/ml) was either loaded alone or was incubated with placebo calcium phosphate nanoparticles overnight at 10 °C to adsorb it on the particle surface and then loaded. In both cases control experiments involving treatment with DNaseI for 30 min prior to loading were also conducted.

2.8. In vitro transfection studies in mammalian (Jurkat) cell-line

Jurkat cells were grown in RPMI-1640 medium containing 1% fetal calf serum and 10% streptomycin and penicillin. For transfection experiments, Jurkat cells were grown overnight. Cells were then centrifuged and resuspended in RPMI-1640 and cells were diluted to concentration of 10^7 cells/ml. Three hundred and fifty microliter of this cell-suspension was added to each well of 24 well-plate and kept for 4 h. After checking the cell viability using trypan blue exclusion, 1 μg of DNA/well was added and kept for 36 h. After 36 h, cells were lysed with lysis buffer containing 250 mM Tris–HCl (pH 7.4) and 0.5% Triton X-100. Ten microliter of X-Gal solution (Promega, USA) was added to each well. After 24 h, the reaction was stopped by adding 50 μl of 1 M sodium carbonate solution. The blue color developed was measured spectrophotometrically at 403 nm (λ_{max}). The quantity of β-galactosidase produced was calculated from a calibration curve (amount of β-galactosidase versus optical density at 403 nm).

2.9. In vivo expression of β-galactosidase in different body tissues after administration of pSVβGal plasmid DNA encapsulated in calcium phosphate nanoparticles

Young Swiss albino mice (average weight 15–20 g) were injected intraperitoneally (i.p.) with 50 μl sterile aqueous suspension of calcium phosphate nanoparticles (filtered through 0.2 μm Millipore filter) encapsulating 10 μg of pSVβGal DNA. Naked DNA (10 μg) was injected to a different set of animals. Mice were sacrificed 72 and 96 h later, and lungs, spleen and liver were harvested and homogenized in cold (0.25 M) Tris–HCl buffer, pH 7.4. Extracts were prepared by centrifugation ($12\,000 \times g$), and assayed for protein using Lowry's method (Lowry et al., 1951).

Each tissue was normalized for protein and assayed for β-galactosidase activity using the following procedure. To 200 μl of extract 4 μl X-Gal (Promega, USA) was added, followed by overnight incubation at 37 °C in tightly capped tubes. Reaction was terminated by addition of 100 μl 1 M Na_2CO_3 and absorbance read at 415 nm. The enzyme activity detected was over and above that of the background endogenous activity. Enzyme activity was expressed as mU/mg tissue protein. In another group, calcium phosphate nanoparticles encapsulating plasmid DNA (10 μg) was injected into the *tibialis* muscle bundle of mice. Animals were sacrificed at pre-determined time intervals; muscle, liver, lung and spleen were removed. A different set received 10 μg of naked DNA injected intramuscularly (i.m.). Following

homogenization and centrifugation protein and enzyme activity was determined. Six animals were used for each experimental set.

2.10. Statistical analysis (Student's t-test)

Data have been represented as the mean of six individual observations, with the standard error of mean. Significance has been calculated using Student's *t*-test.

3. Results and discussion

The strategy of the preparation of calcium phosphate nanoparticles containing plasmid DNA involves the co-precipitation of calcium phosphate and DNA in the aqueous core of the reverse micellar droplets. About 85% of DNA added in the reverse micelles was found to enter the matrices of the nanoparticles. This was determined from the encapsulation efficiency ($E\%$) study by estimating the amount of free DNA present in the micellar phase after centrifugation. Therefore, in each 10 ml CaPi–DNA suspension in aqueous buffer, which was originally supposed to have 8.0 μg of DNA, we have 1.0 μg DNA left in the micellar phase indicating that 7.0 μg of DNA has been loaded in the total amount of calcium phosphate nanoparticles. The size of the particles obtained was determined using dynamic light scattering (DLS) measurements. The average size of the particles was found to be 80 nm in diameter (Fig. 1a) with low polydispersity. Water-core size of reverse micellar droplets as well as reaction temperature, concentration of calcium ions and speed of mixing of the both microemulsion systems influence the size of the nanoparticles. Transmission electron micrograph showed that the particles were spherical in shape having solid core, with occasional clustering (Fig. 1b).

The crystallinity of the calcium phosphate nanoparticles was studied by means of an X-ray powder diffractometry which indicated the formation of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, (HAP), crystals as evidenced from the characteristic peak at $2\theta = 31.8^\circ$ (Shimabayashi et al., 1995). In the case of the DNA doped nanoparticles the crystallinity was totally lost (data not shown). It is assumed that the doped molecules inhibited the crystallization of the entrapping matrix.

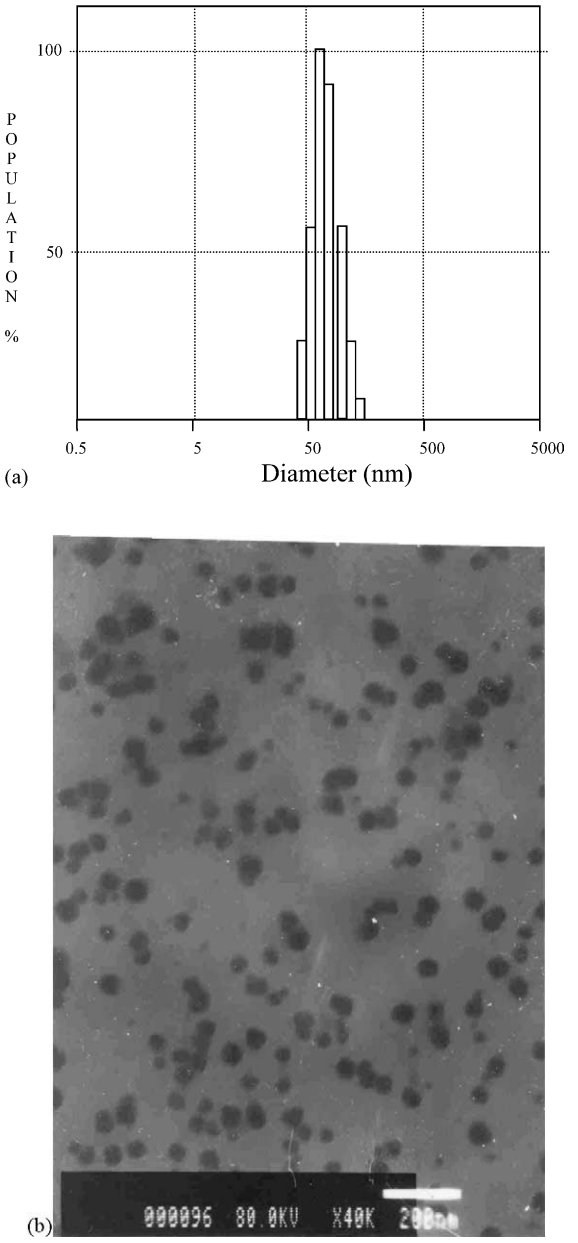

Fig. 1. (a) Particle size of DNA encapsulated calcium phosphate nanoparticles as measured by Photon Correlation Spectroscopy (DLS). (b) Transmission electron micrograph (TEM) of DNA doped calcium phosphate nanoparticles.

One of the primary reasons for the low transfection efficiency obtained with non-viral vectors, including the precipitated calcium phosphate is the partial protection of the plasmid DNA by the encapsulating material as well as low cell surface accumulation of the plasmid (Luo and Saltzmann, 2000). Such partial protection makes the DNA highly susceptible to aggressive DNase attack in the body as well as inside the cell. In order to check the level of protection being offered to the encapsulated DNA, we subjected the nanoparticles to DNaseI treatment (see experimental part for details) for 30 min followed by electrophoresis on 1% agarose gel (Fig. 2). We found that while naked plasmid DNA (pUC19) moved at its usual position in the gel, pUC19 encapsulated in the matrix of the nanoparticle was right at the top of the gel and hardly moved. Moreover, while naked pUC19 was completely digested by DNaseI treatment, encapsulated pUC19 was not degraded. As expected, there is hardly any protection from DNase degradation for the naked plasmid DNA mixed with the placebo nanoparticles (Fig. 2, lane 7). In this case, we find that the level of protection offered to the DNA was extremely low and the DNA was highly prone to total degradation by DNaseI. These results clearly demonstrate that we have been successful for the first time in encapsulating the DNA in the rigid matrix of the calcium phosphate nanoparticles and have been able to protect it from external DNase environment. This data, further boosted by the fact that calcium phosphate is relatively non-toxic at the levels used (He et al., 2000), indicates that the particles may be used for safe and efficient delivery of the encapsulated DNA in vivo.

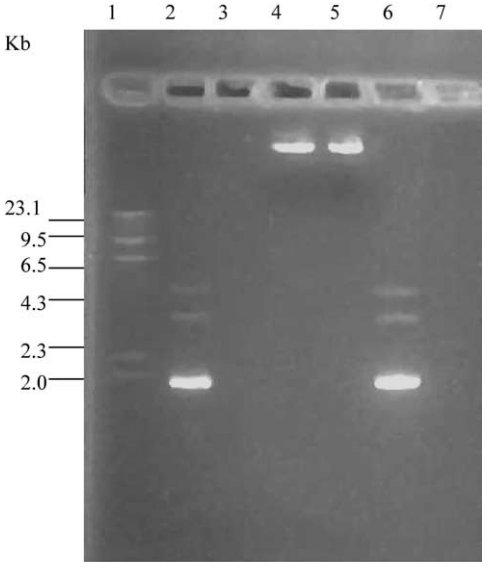

Fig. 2. Agarose (1%) gel electrophoresis of free, entrapped and adsorbed pUC19 DNA. Lane1: M.W. marker—5 μl of λ DNA digested with *Hin*d III enzyme. Lane 2: Free pUC19 DNA. Lane 3: pUC19 DNA treated with DNaseI. Lane 4: Calcium phosphate nanoparticles containing entrapped pUC19 DNA. Lane 5: Calcium phosphate nanoparticles containing entrapped pUC19 treated with DNaseI. Lane 6: pUC19 DNA adsorbed on void calcium phosphate nanoparticles. Lane 7: pUC19 DNA adsorbed on void calcium phosphate nanoparticles and then treated with DNaseI.

Use of calcium phosphate alone or in combination with other vectors exerts its positive effect on the gene transfer by stimulating cellular uptake of DNA in a process involving either endocytosis of the membrane bound DNA complex or enhanced permeability of the plasma membrane to facilitate DNA entry and, therefore, the possible benefits of nanoparticle mediated gene transfer by co-delivery of calcium ions have already been reported (Orrantia and Chang, 1990). Calcium phosphate nanoparticles encapsulating DNA have been observed to achieve this benefit when these were added to Jurkat cell line in vitro (Fig. 3). The plasmid DNA used in this case was pSVβgal which carries the reporter gene coding for the enzyme β-galactosidase (Zhang et al., 2001). The transfection efficiency obtained using these nanoparticles was nearly comparable to that obtained using commercially available transfecting reagent (Superfect, obtained from Promega, USA) and was higher than that obtained using calcium phosphate DNA co-precipitate. The higher transfection efficiency of these nanoparticles compared to precipitated calcium phosphate could be attributed to the ultra-low size of the carrier particles combined with the effect of calcium ion mediated endocytosis.

To assess the potential utility of calcium phosphate nanoparticle mediated gene delivery in the animal, in general, and to specific organs of the animal in particular, we have used the murine model. Experiments were conducted on young Swiss albino mice, and we studied the local gene

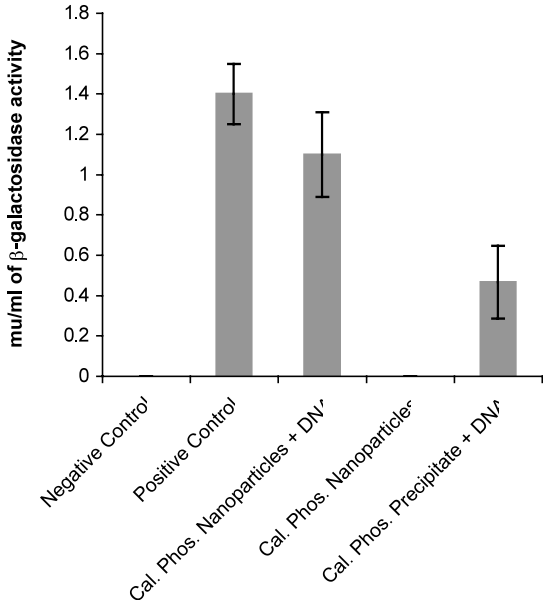

Fig. 3. In vitro transfection studies in mammalian (Jurkat) cell-line.

expression, as well as expression in different body tissues. Interestingly, both i.m. and i.p. administration of encapsulated pSVβgal resulted in expression of the β-galactosidase enzyme in major organs of the body (Fig. 4A and B). Local expression was observed in the *tibialis* muscle bundle. The tissue expression of the injected naked DNA was found to be either less than or equivalent to that of DNA encapsulated in nanoparticles. The observation where lower expression of naked DNA was seen could possibly be due to the vulnerability of the naked DNA to degradation by DNase present extracellularly. The muscle tissues are relatively dense without a lot of space for particles to diffuse through and, therefore, the particle uptake is expected to have a lower rate (Fewell et al., 2001; Smith and Nordstrom, 2000) compared to the rate at which the nanoparticles reach the systemic circulation, primarily to the lung which is encountered first, and subsequently by other tissues (Mahato et al., 1997). As a result, the lung cells largely take up these untargeted calcium phosphate nanoparticles, although expressions are observed in other tissues also. From the biodistribution of these DNA doped calcium phosphate nanoparticles it is apparent that these non-viral carriers may present a novel delivery system for genetic material to be transported into the body.

The liver is an important target for gene therapy, because of its large size and protein synthetic capacity. Moreover, there is a need to target genes to the liver for treatment of diseases involving defects in members of segmental enzymatic pathways that are unique to the organ. Models for hepatic gene delivery have been developed using viral vectors (Futagawa et al., 2000; Zhu et al., 1997; Hofmann et al., 1995), virosomes (Ramani et al., 1997) and other non-viral vectors (Bandopadhyay et al., 1997; Remy et al., 1995), but all of these methods have important limitations. In our studies we have explored the potential

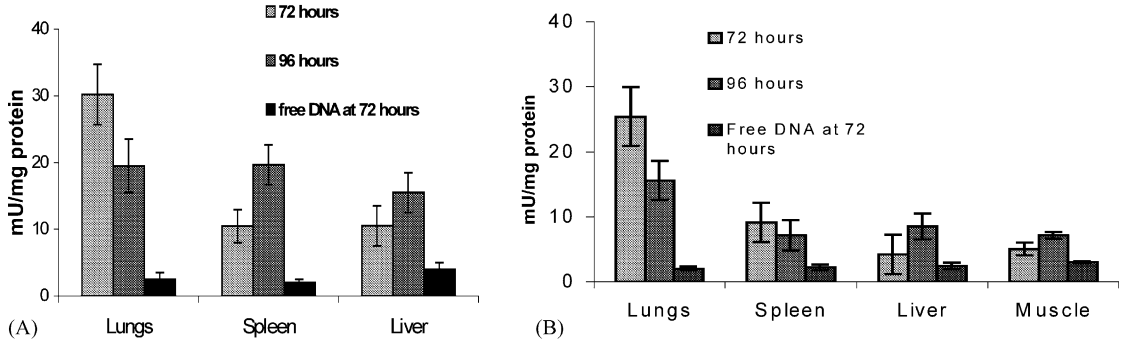

Fig. 4. β-Galactosidase expression in different body tissues after in vivo administration of pSVβGal plasmid DNA encapsulated in calcium phosphate nanoparticles. (A) β-galactosidase expression in lungs, liver and spleen after i.p. injection of nanoparticles encapsulating DNA. (B) Enzyme expression in lungs, liver, spleen, and muscle after i.m. injection of nanoparticles.

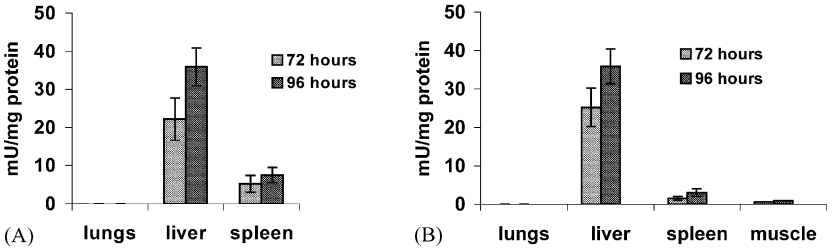

Fig. 5. β-Galactosidase expression in vivo after administration of galactopyranoside-tagged calcium phosphate nanoparticles encapsulating pSVβGal plasmid DNA. (A) β-Galactosidase expression in lungs, liver and spleen after i.p. injection of the tagged nanoparticles encapsulating DNA. (B) Enzyme expression in lungs, liver, spleen and muscle after i.m. injection of tagged nanoparticles.

use of calcium phosphate nanoparticles as vectors for hepatic gene transfer. Targeted delivery of genes to liver by attaching targetable ligand on the surface of the non-viral carriers is possible. Nanoparticles were incubated with a highly adhesive polymer like PAA, followed by dialysis to remove excess polymer. The PAA molecules adhered on the surface of the calcium phosphate nanoparticles were further modified by conjugating the carboxylic groups with *p*-aminophenyl-1-thio-β-D-galactopyranoside (PAG) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI). The galactopyranoside moiety serves as a surface ligand for recognizing asialoglycoprotein receptor on liver cells (Nishikawa et al., 2000). The tagged nanoparticles have an average size of 85 nm as measured by QELS (data not shown) and showed preferential expression in the liver tissue relative to lung, spleen and muscle (Fig. 5A and B). On i.m. administration of these surface modified calcium phosphate nanoparticles doped with plasmid DNA the muscle uptake has been found to be insignificant compared to that of liver cells, which have been targeted. The lung uptake during systemic circulation of these surface modified particles is also practically negligible as compared to unmodified calcium phosphate nanoparticles having no surface ligand. Attachment of galactose moiety onto the particle surface has increased the targetability of the particles to the liver cells more efficiently. As a matter of fact, the liver uptake has been found to be increased to about 400–500% compared to that observed with unmodified calcium phosphate nanoparticles. Liver uptake has been found to be more through intraperitoneal route than through i.m. route. These observations suggest redistribution of genetic material in relation to the particle surface characteristics. Suitable surface modification of the nanoparticles also provides opportunities for site-specific gene delivery.

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