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Research paper

Chitosan microparticles containing plasmid DNA as potential oral gene delivery system

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Abstract

The potential of chitosan as a polycationic gene carrier for oral administration has been explored since 1990s. Chitosan has been shown to effectively bind DNA in saline or acetic acid solution and protect DNA from nuclease degradation. In this study, pDNA (plasmid DNA) was encapsulated in chitosan microparticles. Chitosan–DNA microparticles were prepared using a complex coacervation process and stability of plasmid DNA was investigated in this complex. The chitosan–DNA microparticles could protect the encapsulated plasmid DNA from nuclease degradation. Release of pDNA from microparticles was studied in simulated gastric, simulated intestinal medium and acidic PBS (phosphate buffer saline) (pH 4.5) buffer at 37 °C, and released pDNA was assayed spectrophotometrically. In vitro release of pDNA from chitosan microparticles was dependent on pH, as the pH of the release medium increased release profile decreased. In in vivo-animal studies blue color was observed with X-gal (4-chloro-5-bromo-3-indolyl-β-galactosidase) staining of histological stomach and small intestine sections after oral administration of pDNA–chitosan microparticles as an indicator of exogeneous gene expression.

Keywords: Chitosan; Microparticles; Gene therapy; Oral gene delivery; Plasmid DNA; Beta-galactosidase expression

1. Introduction

The development of appropriate vehicles to deliver new biological macromolecules is a new challenge for pharmaceutical scientists. For efficient gene delivery, plasmid DNA must be introduced into targeted cells, transcribed and the genetic information ultimately translated into corresponding protein [1]. Although viral gene delivery systems yield high transfection efficiency over a wide range of cell targets [2], they have major drawbacks, such as virally induced inflammatory responses and oncogenic effects [3].

To overcome these disadvantages, gene delivery studies have also been planned for development of non-viral gene delivery systems. They can all be administered repeatedly with minimal host immune response, and are stable in storage, targetable, and easily prepared [4]. Cationic polymers have been shown as promising carriers among the non-viral gene delivery systems [5]. Compared with other non-viral gene delivery systems, polycation-DNA complexes are generally more stable. A number of cationic polymers have been investigated as gene carriers [6–8]. An ideal polymeric gene carrier should have high efficacy of gene transfer, targeting ability and good biocompatibility. High stability, especially in the lyophilized form, is also thought to be an important feature to apply genes as pharmaceuticals [9].

Transfection is hindered by (A) targeting the delivery system to the target cell, (B) transport through the cell membrane, (C) degradation in endolysosomes, and (D) intracellular trafficking of plasmid DNA into the nucleus [1]. Chitosan–pDNA complexes appeared to have ability to overcome three major obstacles for transfection, i.e. cell uptake, endosomal release and nuclear localization [10]. Besides many nanoparticle studies [8,9], plasmid DNA–chitosan complexes were also studied in the form of self aggregates [11,12], emulsions [13] and microspheres [14] for transfection of eucaryotic cells showed that the chitosan can be used in many forms as a promising gene carrier.

In recent years, the potential of chitosan as a polycationic gene carrier has been explored in several research groups [8]. Chitosan [β(1–4)2-amino-2-deoxy-D-glucose] is obtained by

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alkaline deacetylation of chitin. Chitosan molecule is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine [15,16]. Chitosans differ in degree of *N*-acetylation (40–98%) and molecular weight (50–2000 kDa) [17]. Mucoadhesive property of chitosan potentially permit a sustained interaction of the macromolecule to be 'delivered' with the membrane epithelia, promoting more efficient uptake [18]. Chitosan is a biodegradable and non-toxic polysaccharide [19]. Due to its good biocompatability and toxicity profile, it has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as biomedical material for artificial skin and wound healing bandage applications [20].

Chitosan was first described as a delivery system for plasmids by Mumper et al. [21]. In the other examples, small plasmid/chitosan nanoparticles (200–300 nm) were prepared by complex coacervation method by Roy et al. [8]. Mao et al. [9] modified chitosan nanospheres with transferrin and PEG, but no significant enhancement in transfection efficiency was observed.

The oral delivery of peptide, protein, vaccine and nucleic acid-based products is the great challenge in the drug delivery industry. Oral delivery is attractive due to the factors such as ease of administration, and improved patient convenience and compliance, thereby reducing overall healthcare costs.

Chitosan has been used successfully to deliver a reporter gene (encoding chloramphenicol acetyl transferase) orally to enterocytes, Peyer's patches and mesenteric lymph nodes [22]. In another study mice fed with chitosan–DNA particles by mixing their fed, expressed the reporter gene *lacZ* (encoding bacterial β-galactosidase) 5 days after oral administration [8]. This study also showed that orally administered chitosan–DNA complexes can stimulate an immune response to the principal peanut allergen Arah-2.

In this study, we have prepared chitosan–pDNA microparticles and examined in vitro stability and in vivo efficiency of encapsulated DNA. Release profiles of DNA from chitosan–pDNA microparticles in simulated gastric, simulated intestinal medium and acidic PBS buffer were observed. In vivo transfection of chitosan microparticles containing plasmid DNA was investigated. To assess the expression and distribution of transduced genes after oral DNA delivery, pDNA chitosan microparticles were applied to mice orally with a special intragastric drill. Then mice were fed with standard diet. The expression of Lac Z gene and activity of α-galactosidase enzyme in stomach and small intestine tissues were detected using X-gal solution at acidic and alkaline pH. At acidic pH β-galactosidase endogenous activity, and at alkaline pH β-galactosidase exogenous activity were observed.

2. Materials and methods

2.1. Materials

Chitosan H (MW: 1400 kDa, deacetylation degree is 80%) was purchased from MGF Co. Ltd (Japan). Plasmid pnlacF was kindly contributed by R. Palmiter (Howard Hughes Medical Institute Research Laboratories, University of Washington, USA). pnlacF (6900 bp) contains lacZ gene which encodes β-galactosidase, ampicillin resistance gene and SV 40 early promoter.

Chitosanase and DNase I were obtained from Sigma–Aldrich Corp. (USA); restriction enzymes BamHI and EcoRI were obtained form Promega Corp. (USA); X-Gal (4-chloro-5-bromo-3-indolyl-β-galactosidase) was obtained from Bio-Rad Lab. Inc., (USA); mice (4–6 weeks, 20–25 g) were obtained from Experimental Animals Department of Hacettepe University (Ankara, Turkey). All other chemicals were obtained from Sigma–Aldrich Corp. (USA).

2.2. Amplification and purification of plasmid DNA

pnlacF was amplified in JM 109 strain of *E. coli* and extracted by alkaline-lysis technique [23] and purified by precipitation with ethanol. Then the precipitate was dissolved in TRIS-acetate-EDTA buffer and concentration of DNA was determined UV-spectrophotometrically at 260 nm. The integrity of isolated plasmid DNA was observed by agarose gel electrophoresis (0.8%, w/v).

2.3. Analysis of DNA structure

The DNA structure was analyzed by digesting with BamHI and EcoRI restriction enzymes. The digestion was performed at 37 °C for 4 h. Electrophoretic mobility of the digested DNA was analyzed by 0.8% agarose gel electrophoresis. Samples were run for 60 min at 110 V in TRIS-acetate-EDTA buffer system (pH 8.0) and visualized using ethidium bromide staining.

Transilluminator and imaging system (Alpha-Innotech Corp., USA) were used to detect DNA and to take photographs under UV light.

2.4. Preparation and characterization of chitosan–DNA microparticles

Plasmid loaded chitosan microparticles were prepared by complex coacervation method [14,24]. Sodium sulfate solution (20%, w/v) containing plasmid was dropped into the chitosan solution (0.25% in 0.1 M acetate buffer, pH 4.5) and stirred (Heidoph, Germany) at 700 rpm for 40 min. Formed particles in the suspension were frozen at −20 °C without separation, lyophilized and stored at 4 °C after freeze–drying (Vitris Freezemobile 5). A gel retardation assay was applied for monitoring pDNA–chitosan complex formation ratios by loading samples onto 0.8% agarose gel. After staining in ethidium bromide (EtBr) gel photographs were taken under UV light. Size of microparticles was determined using an ocular micrometer in a light microscope (Leica, Germany). Leica DC 300 digital camera is used and digitalized microscope images displayed on the PC screen. Transmission Electron Microscopy (TEM) was used to determine particle morphology and confirm particle size range. TEM equipped with a 100 kV Jeol JEM-100 CX with 2A resolving power.

2.5. Stability of plasmid in chitosan microparticles

Stability of plasmid DNA from DNAse degradation was examined using DNase I. Naked DNA (3 μg in 10 μL of 20% of Na_2SO_4) and microparticle suspension (10 μL, equivalent to 3 μg DNA) were incubated with DNAse I (4 or 40 μg/mL) at neutral pH for 15 min at 37 °C [9]. The reaction was stopped by adding 0.1 M EDTA at pH 8. After this treatment microparticles were subjected to chitosanase digestion for 4 h at 37 °C. The integrity of pDNA was then analyzed using 0.8% agarose gel electrophoresis.

Stability of pDNA in chitosan microparticles was also determined by analyzing lyophilized forms which were kept at +4, +25, and +37 °C for 1 month. Examples were then resuspended in water and digested by chitosanase for 4 h at 37 °C. The stability of plasmid DNA in microparticles was visualized using EtBr staining and analyzed using 0.8% agarose gel electrophoresis.

2.6. In vitro release studies

In vitro release studies were done in simulated mouse gastric (pH 2.1) [25] and simulated mouse intestinal medium (pH 6.47) [26] and in acidic PBS medium at 37 °C. The released plasmid DNA was determined using UV-spectrophotometer at 260 nm. Linear range of quantification between 1 and 50 μg/mL was determined in every dissolution medium ($r^2 = 0.9998$ for simulated gastric medium, 0.9995 for simulated intestinal medium and 0.9930 for acidic PBS medium). In order to eliminate excipient effects blank solutions were used including the same excipients. For this purpose, lyophilized chitosan–pDNA microparticles were dispersed in 4 mL of medium, and 800 μL of dissolution medium was taken at fixed periods of time. Microparticles containing 100 μg pDNA were added to the dissolution medium and agitated in a shaking water bath at a rate of 50 per min. After each sampling, 800 μL of fresh medium was added into dissolution medium. Released samples were examined with agarose gel electrophoresis.

2.7. In vivo animal studies

Animals were divided into five groups as seen in Table 1. Swiss albino mice of both sexes were housed for a 6 days period in proper conditions and on diet. Preparations were applied orally by using a special intragastric drill (Fig. 1).

Table 1
In vivo animal study groups

Group no.	Number of animals	Administered preparation	Total pDNA dose
I	5	Control (no preparation only animals feed)	–
II	4 (1 died)	Chitosan microparticles	–
III	5	Naked pDNA	50 μg
IV	5	Chitosan–pDNA microparticles	50 μg
V	5	Chitosan–pDNA microparticles	100 μg

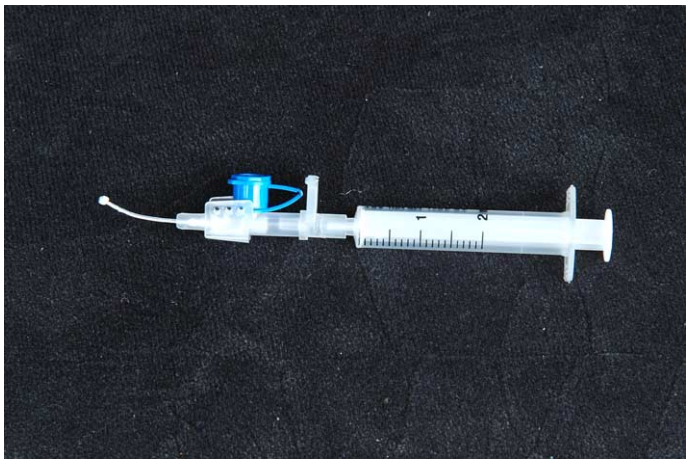

Fig. 1. Special intragastric drill.

Animal experiments were performed with the permission of the Animal Ethical Commission of Hacettepe University (Approval no: B.30.2.HAC.0.01.00.05).

Mice were fed with chitosan–pDNA microparticles containing Lac-Z gene and then the expression of bacterial β-galactosidase in the stomach and intestine were determined.

Six days after application, the mice were sacrificed, and their stomachs and small intestines surgically removed. Another studies indicated that mice fed with chitosan–DNA particles expressed the reporter gene Lac-Z, 3 and 5 days after administration [8,27]. Removed organs were rinsed in phosphate-buffered saline (PBS) and minced, and minces were fixed for 30 min at room temperature in 0.5% glutaraldehyde in PBS, pH 7.3 containing 1 mM $MgCl_2$. Fixed organ minces were divided into two equal portions and incubated with 0.2% 4-chloro-5-bromo-3-indolyl-β-galactosidase (X-Gal) in 100 mM TRIS buffer containing 5 mM ferriferro-cyanide and 2 mM $MgCl_2$ at acidic (4.5) and alkaline (7.5) pHs [28]. After staining overnight at 37 °C with X-gal solution, X-Gal was removed, minces were washed with PBS and post-fixed in 10% formalin solution. Tissues were embedded in paraffin wax, sectioned and mounted on glass slides for histopathological examination. Histological sections were evaluated by three investigators using a semi-quantitative rating scale as seen in Table 2. This rating scale was used for determination of both endogenous and exogenous β-gal activity. For each histological section, the entire microscopic field was evaluated for evidence of β-gal activity.

Table 2
Semi-quantitative rating scale of β-galactosidase activity in tissues

Numbers	Coloring of tissues
4	Coloring of 70–100% of tissues
3	Coloring of 40–70% of tissues
2	Coloring of 10–40% of tissues
1	Coloring of $\leq$10% of tissues
0	No color changes

3. Results and discussion

Complex coacervates of pDNA and chitosan could be used as oral delivery vehicle in gene therapy and vaccination studies [8,10]. Chitosan–pDNA formulations were offered useful oral gene delivery systems because of adhesive and transport properties of chitosan in the gastrointestinal tract [17]. In this study, we prepared complex coacervate microparticles (0.66–1.35 μm) from chitosan and pDNA by using sodium sulphate as a coacervation agent. Lac-Z reporter gene containing plasmid, pnlacF is used to detect β-galactosidase expression in the cells of stomach and small intestine.

3.1. Analysis of DNA structure

Agarose gel electrophoresis photograph of pnlacF and fragments after restriction enzyme treatments were seen in Fig. 2a and b.

In Fig. 2a, supercoiled and open circular forms of pnlacF is seen. In Fig. 2b, restriction enzyme fragments were seen. BamHI cuts pnlacF (6.9 kbp) as two identical pieces seen in the same place; EcoRI cuts pnlacF as two different sized bands as described in the data sheet of plasmid.

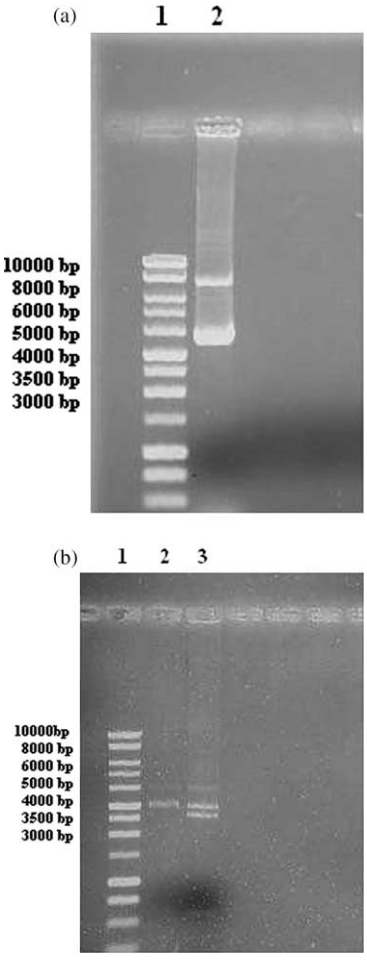

Fig. 2. (a) Naked pnlacF. *Lane 1*. Marker, 1 kb DNA ladder; *Lane 2*. Naked pnlacF. (b) pnlacF digested with restriction enzymes. *Lane 1*. Marker, 1 kb DNA ladder; *Lane 2*. pnlacF digested with BamHI. *Lane 3*. pnlacF digested with EcoRI.

3.2. Characterization of chitosan–DNA microparticles

Agarose gel electrophoresis photographs of pDNA–chitosan complex formation at different ratios of pnlacF and chitosan by using gel retardation assay were seen in Fig. 3a and b.

Empty microparticles were about 0.66–1.32 μm, loaded microparticles were about 1.32–2.64 μm in size. TEM

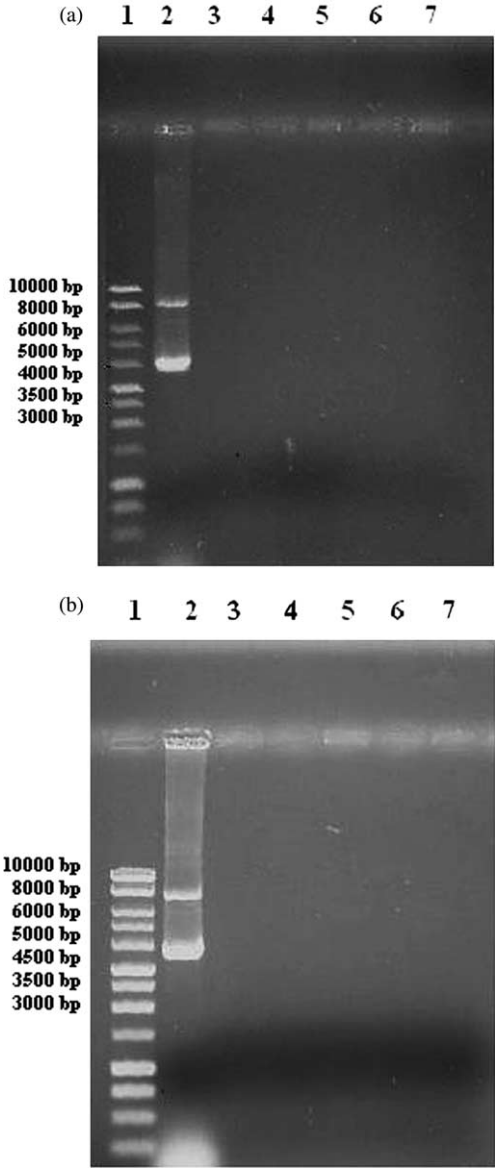

Fig. 3. A gel retardation assay for monitoring pDNA–chitosan complex formation at different ratios. (a) *Lane 1*. Marker, 1 kb DNA ladder; *Lane 2*. Naked pnlacF; *Lane 3*. pnlacF–chitosan complex (1:0.0025); *Lane 4*. pnlacF–chitosan complex (1:0.0125); *Lane 5*. pnlacF–chitosan complex (1:0.025); *Lane 6*. pnlacF–chitosan complex (1:0.05); *Lane 7*. pnlacF–chitosan complex (1:0.125). (b) *Lane 1*. Marker, 1 kb DNA ladder; *Lane 2*. Naked pnlacF; *Lane 3*. pnlacF–chitosan complex (1:0.5); *Lane 4*. pnlacF–chitosan complex (1:1.25); *Lane 5*. pnlacF–chitosan complex (1:2.5); *Lane 6*. pnlacF–chitosan complex (1:5); *Lane 7*. pnlacF–chitosan complex (1:12.5).

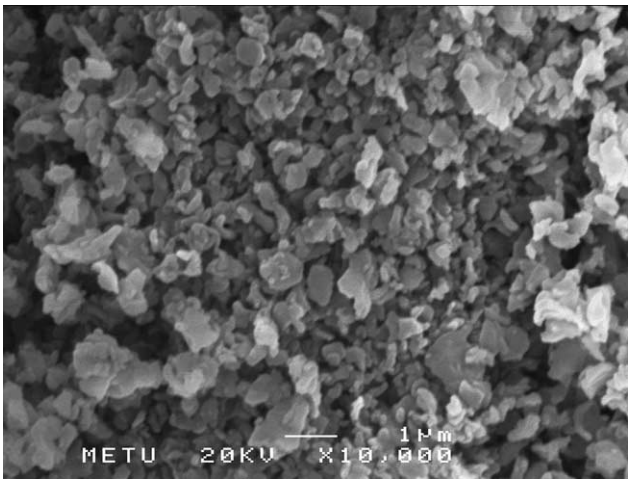

Fig. 4. Transmission electron micrograph of microparticles.

photographs of microparticles are seen in Fig. 4 to confirm these sizes.

3.3. Stability of plasmid in chitosan microparticles

The ability of the chitosan to form complex with DNA and to stabilize pDNA from endonuclease degradation were studied by using agarose gel electrophoresis. pDNA and pDNA–chitosan microparticles were digested with DNase I (25 U/ml) at 37 °C. In our preliminary studies, 15 min is found appropriate for DNase I digestion. Naked plasmid DNA displayed different form from the natural conformation after DNase I treatment. There was also smearing on the gel image of naked DNA (lane 5 of Fig. 5). In contrast, plasmid DNA recovered from microparticles after the same treatment protected from degradation and gel migration images were not changed (lanes 7 and 9 of Fig. 5).

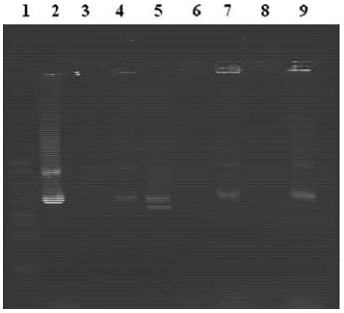

Fig. 5. Electrophoretic mobility analysis of chitosan–DNA microparticles following DNAse I digestion. Naked DNA and microparticles were both incubated with different concentrations of DNase I for 15 min at 37 °C. The reaction was stopped by addition of 100 mM EDTA. These microparticles were then subjected to chitosanase digestion. All examples were run on 0.8% agarose gel and stained with ethidium bromide. *Lane 1*: marker, 1 kb DNA ladder; *Lane 2*: naked pnlacF; *Lane 3*: microparticles; *Lane 4*: microparticles digested with chitosanase; *Lane 5*: pnlacF + DNase I (4 (μg/mL); *Lane 6*: microparticles + DNase I (4 (μg/mL); *Lane 7*: microparticles + DNase I (4 (μg/mL) digested with chitosanase; *Lane 8*: microparticles + DNase I (40 (μg/mL); *Lane 9*: microparticles + DNase I (40 (μg/mL) digested with chitosanase.

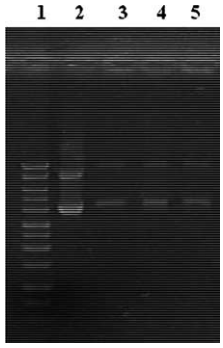

Fig. 6. Electrophoretic mobility of chitosan–DNA microparticles which were kept at +4, +25, and +37 °C for 1 month. *Lane 1*: marker, 1 kb DNA ladder; *Lane 2*: fresh chitosan–DNA microparticles digested with chitosanase; *Lane 3*: chitosan–DNA microparticles kept at +37 °C for 1 month and digested with chitosanase; *Lane 4*: chitosan–DNA microparticles kept at +25 °C for 1 month and digested with chitosanase; *Lane 5*: chitosan–DNA microparticles kept at +4 °C for 1 month and digested with chitosanase.

This suggested that at physiological conditions where the nuclease concentration is markedly lower than the tested concentrations, such a formulation should render protection of the plasmid. In the presence of different enzymes data may look differently but chitosans have the ability to protect DNA against nuclease degradation. Richardson et al. [29] used DNAse II to show the stability of pDNA in the complex. Proposed mechanism for protection is explained with complexation induced changes in DNA tertiary structure causing steric hinderance. Lyophilized forms of pDNA:chitosan microparticles were stored at +4, +25, and +37 °C for 1 month. Lyophilization was performed without using cryoprotectant. Examples were then analyzed for pDNA stability in chitosan microparticles by agarose gel electrophoresis (Fig. 6).

As seen in Fig. 6, in all freeze–dried chitosan–pDNA microparticles, pDNA kept its stability during the 1 month at higher and lower temperatures.

3.4. In vitro release studies

Release profiles of pnlacF loaded chitosan microparticles in simulated gastric (pH 2.1), simulated intestinal (pH 6.47) and acidic PBS (pH 4.5) mediums are given in Fig. 7. An initial burst effect was detected followed by a plateau. Rapid burst might be related to DNA adsorbed onto the surface of the particles.

In the gastric and acidic PBS mediums, pDNA was released from chitosan microparticles in shorter periods than in the intestinal medium, and released amounts was high (approximately 85 and 100%). In intestinal medium, the released pDNA amount was lower (approximately 30%) than the acidic medium. In vitro release of plasmid DNA from chitosan microparticles was dependent on pH, as the pH of the release medium increased, release profile decreased, and vice versa. The differences in release profiles were caused by different solubilities of chitosan in acidic and basic pHs of mediums. Used salts may also be interfered with the solubility of the chitosan as reported by Hezaki et al. [17]. We have used high

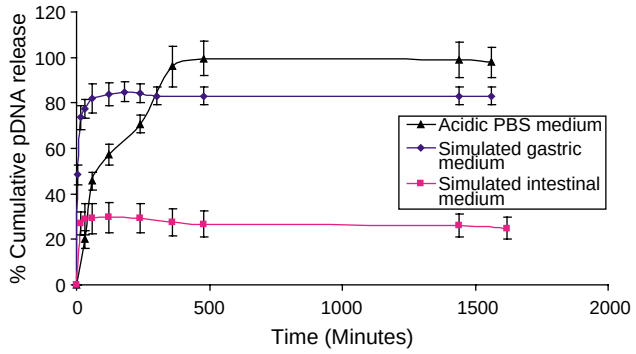

Fig. 7. Release profiles of plasmid DNA-loaded microparticles ($n=3$) in simulated mouse gastric (pH 2.1), simulated mouse intestinal (pH 6.47) and acidic PBS (pH 4.5) mediums.

molecular weight chitosan with high degree of deacetylation. Highly deacetylated chitosans are soluble up to pH 6.5 [17]. Glucosamine units of chitosan shows high density of amine groups and requires pH values less than 6 to be soluble [1]. In acidic medium amino groups of the polymer protonated resulting in a soluble, positively charged polysaccharide leading to faster swelling.

3.5. In vivo animal studies

It has been discovered that genes can be delivered to and can transfect epithelial cells when administered orally [8]. According to the proposed mechanism, there is an initial interaction between the positively charged chitosan–DNA complex and the negatively charged heparan sulphate proteoglycans on the cell membrane [30]. Erbacher et al. [11] monitored cell binding and entry via TEM observations. After adhesion, complexes are taken up by endocytosis. Roy et al. confirmed that 1 h post-incubation chitosan–DNA complexes had been endocytosed [8]. Complexes were also observed to accumulate in the nucleus. Ishii et al. investigated the transfection mechanism of 5-7μm pDNA–chitosan complexes which adhere to the cell surface and endocytosed [10].

Studies have shown that microparticles are able to transport genes to Peyer's patches through M cells and protect DNA from gastric degradation [12]. Both nanoparticles and microparticles prepared with chitosan reported to be useful carriers to deliver genes to the epithelial cells [8,10].

In vivo gene expression was investigated with macroscopic and histological observations in the stomach and intestinal tissues after oral administration of pDNA–chitosan

Table 3
Endogenous β-galactosidase activity in stomach and small intestine tissues of mice incubated overnight in X-gal solution at pH 4.5

Groups	Analysing organs and tissues	Staining tissues yields (%)	Semi-quantitative rating scale
I	Stomach	40–70	3
	Small intestine	70–100	4
II	Stomach	70–100	4
	Small intestine	10–40	2
III	Stomach	70–100	4
	Small intestine	70–100	4
IV	Stomach	70–100	4
	Small intestine	70–100	4
V	Stomach	70–100	4
	Small intestine	10–40	2

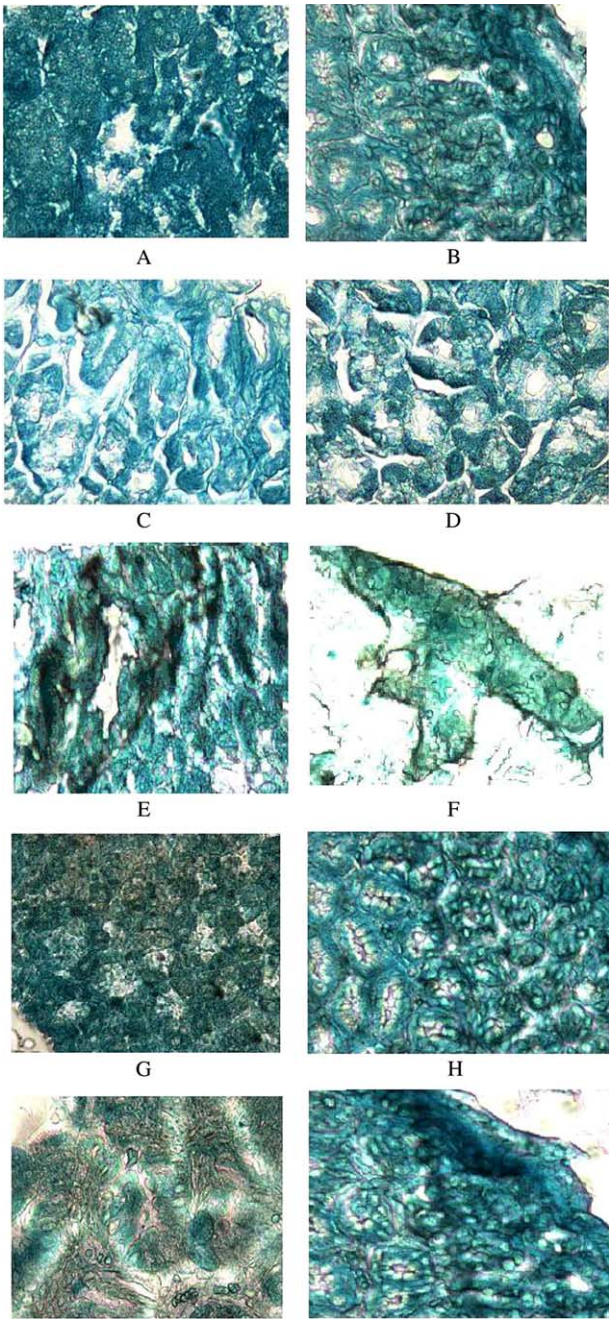

Fig. 8. The stomach and small intestine tissues of mice incubated in X-gal solution at pH 4.5 overnight at 37 °C. (A) Mince of stomach from mouse of group I. (B) Mince of small intestine from mouse of group I. (C) Mince of stomach from mouse of group II. (D) Mince of small intestine from mouse of group II. (E) Mince of stomach from mouse of group III. (F) Mince of small intestine from mouse of group III. (G) mince of stomach from mouse of group IV. (H) Mince of small intestine from mouse of group IV. (I) Mince of stomach from mouse of group V. (J) Mince of small intestine from mouse of group V.

Table 4
Exogenous β-galactosidase activity in stomach and small intestine tissues of mice incubated overnight in X-gal solution at pH 7.5

Groups	Analysing organs and tissues	Staining tissues yields (%)	Semi-quantitative rating scale
I	Stomach	≤10	1
	Small intestine	≤10	1
II	Stomach	≤10	1
	Small intestine	≤10	1
III	Stomach	10–40	2
	Small intestine	≤10	1
IV	Stomach	10–40	2
	Small intestine	10–40	2
V	Stomach	10–40	2
	Small intestine	10–40	2

microparticles. As an indicator of gene expression blue color was observed with X-Gal staining.

Weiss et al. [28] demonstrated that a simple modification of the X-gal method allowed in situ discrimination between endogenous and exogenous β-galactosidase activities in lung. Here, we applied this method for stomach and small intestine tissues. The current study demonstrates that modification of the X-gal techniques as described in the paper of Weiss et al. [31] and Fiering et al. [32] may be applied to stomach and small intestinal tissues to distinguish endogenous and exogenous β-galactosidase activities and exogenous gene expression from endogenous.

Minces of stomach and intestine were incubated with X-gal solution at pH 4.5 and 7.5. At acidic pH, endogenous (mammalian) β-galactosidase activity was detected in all stomach and small intestinal tissues of all animal groups (Table 3, Fig. 8). In contrast, β-galactosidase activity, strongly detected at pH 4.5 in stomach and small intestine tissues of mice in groups I and II, was only weakly evident at pH 7.5. At pH 7.5, perceptible exogenous (bacterial) β-galactosidase activity was detected in stomach and small intestine tissues of mice in groups IV and V. Less evident exogenous β-galactosidase activity was detected in group III in small intestine, but in stomach stain tissue yields were in the same range for groups III–V (Table 4, Fig. 9).

Chitosan–plasmid complexes have a unique property that transfection activity and cell uptake were affected by the pH of the transfection medium [10]. Particles might be positively charged at gastric and deudonal pH, but neutral at intestinal alkaline pH. In the acidic pH gene expression may be resulted from the solubility of the complex. But in the intestine gene expression may be affected by other factors than the solubility. Microflora of the colon has been shown to degrade chitosan [17]. The other factor may be the large surface area for transduction of genes and capillaries very close to the epithelial cell layer which allows the encoded proteins from the epithelium to the systemic circulation [29]. Free and encapsulated plasmid indicated the same score (2) in stomach samples. Free DNA is known to produce gene expression in some tissues like muscle, but delivery systems are required to protect DNA from nucleases for long-term efficient gene transfection.

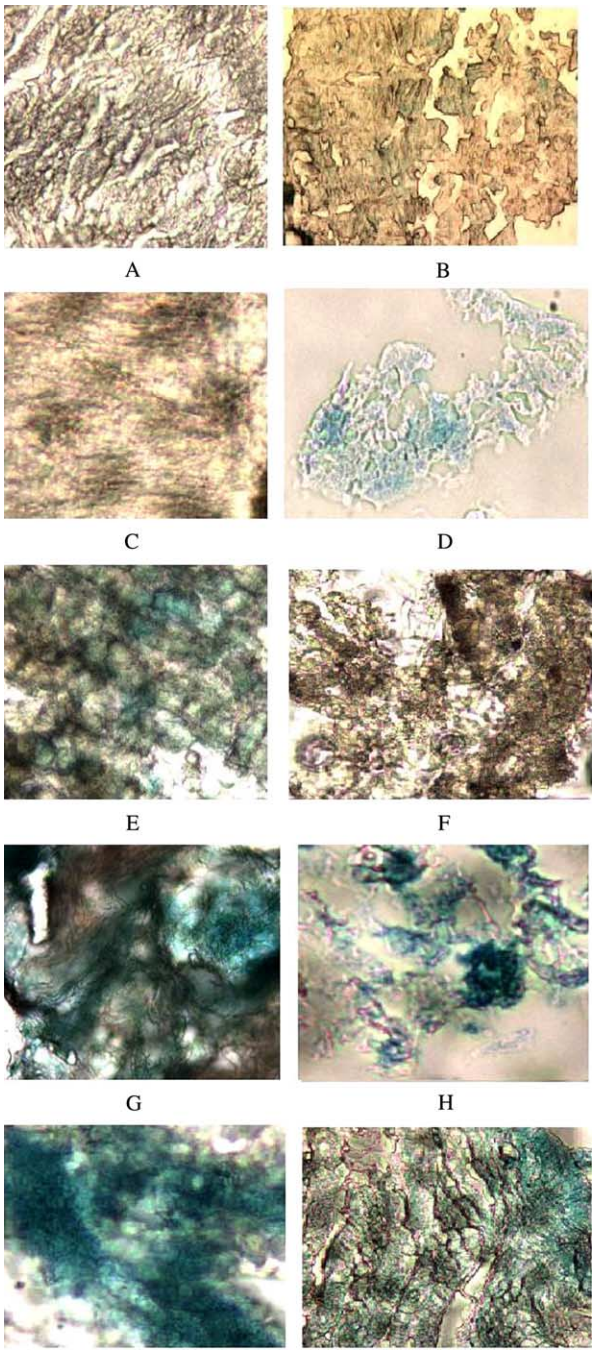

Fig. 9. The stomach and small intestine tissues of mice incubated in X-gal solution at pH 7.5 overnight at +37 °C. (A) Mince of stomach from mouse of group I. (B) Mince of small intestine from mouse of group I. (C) Mince of stomach from mouse of group II. (D) Mince of small intestine from mouse of group II. (E) Mince of stomach from mouse of group III. (F) Mince of small intestine from mouse of group III. (G) Mince of stomach from mouse of group IV. (H) Mince of small intestine from mouse of group IV. (I) Mince of stomach from mouse of group V. (J) Mince of small intestine from mouse of group V.

In the studies of McGregor et al. and Krishenbaum et al., endogenous and exogenous β-galactosidase activities could be distinguished using X-gal at different pHs [33,34]. Our study suggested that endogenous activity tended to be fainter in color

than the dense diffuse blue coloration from exogenous activity. Similar results was reported by Chen et al. [27]. Most of the stomach and small intestine were stained in their study. Both mice and those fed with naked pDNA showed background staining but mice fed with chitosan–DNA nanoparticles showed higher level of gene expression both in stomach and small intestine. These morphological color changes cannot be considered reliable results but seems to be promising.

4. Conclusion

pDNA–chitosan microparticles were prepared using a complex coacervation process under defined conditions. The interaction involved in the complex coacervation was mainly electrostatic. Chitosan microparticles protected pDNA during the storage time in freeze–dried forms and also from nuclease degradation in the mediums.

Release profiles of pDNA from chitosan microparticles in simulated gastric and acidic PBS mediums were higher than in simulated intestinal medium due to the solubility of chitosan in acidic pHs.

In vivo studies demonstrated that gene expression can be achieved by applying the plasmid orally with chitosan microparticles. To determine endogeneous and exogeneous Lac-Z gene expression modified X-Gal techniques can be applied successfully.

We can conclude from these results that pDNA can be encapsulated in the chitosan microparticles without damage using the method described in the study. These pDNA–chitosan microparticles may be a promising method for gene therapy by oral administration with further studies.

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