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Effect of the linear aliphatic amine functionalization on *in vitro* transfection efficiency of chitosan nanoparticles

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ABSTRACT

The aim of this study is to prepare the long linear aliphatic amine pendant group-functionalized chitosan based nanoparticulate gene carrier system with improved properties for the efficient transfection. The amine functionalized chitosan (MChi) was synthesized by using N-(2-hydroxyethyl)ethylenediamine (HE-EDA) and characterized for the first time. The nanoparticles of MChi (nMChi) were prepared by ionic gelation method, and their particle size, polydispersity (PDI), zeta potential (mV), gene binding capacity and cytotoxicity were determined. Green Fluorescent Protein circular plasmid DNA (pEGFN1) loaded nanoparticles (gnMChi) were used in the transfection studies. The results showed that nMChi with a particle size of 102.9 nm and zeta potential of 41.9 ± 5.63 mV was non-toxic, had high transfection efficiency in Human Embryonic Kidney 293 and Primary Ovine Fibroblast cell lines and would be used as an efficient gene carrier system.

1. Introduction

Gene transfer studies started with cell transgenesis *in vitro*, continued with transgenic animal production and recently focused on the gene therapy. Gene therapy not only treats diseases but also aims to carry recombinant genetic material to the nucleus (Xu et al., 2008). However, DNA is not efficiently passed through the cell membrane, and carrier systems could therefore play an important role to deliver the DNA into the cells. So far many viral and non-viral carriers have been used in gene therapy. Viral vectors can provide efficient delivery of a gene to many different cell types. However, some limitations, such as the use of viruses in production, immunogenicity, toxicity and lack of optimization in large-scale production, restrict the use of these systems (Cevher, Sezer, & Çağlar, 2012). Taking into account the limitations of viral based systems, nonviral alternatives have gained significant attention as an alternative.

Non-viral gene delivery systems are divided into two groups as lipophilic and polymeric. These systems are not efficient enough for gene transfer on their own, so they do not have commercial products (Taira, Kataoka, & Niidome, 2005; Wong, Pelet, & Putnam, 2007). Therefore, the researchers have interested in improving the various polymeric vectors to be at least as efficient as viral vectors. At the same time, the common target of these studies is about not to lose their low toxicity (Luo & Saltzman, 2000; Vorhies & Nemunaitis, 2009). The most important advantage of polymeric vectors and polypeptides is their ability to increase their activity by allowing various modifications to improve the release properties of the genetic material on target area (Thomas & Klibanov, 2003).

Chitosan is a promising polymer to prepare non-viral gene delivery systems due to its cationic structure. Chitosan, partially deacetylated product of chitin, is a natural, biocompatible, biodegradable and non-toxic cationic linear polysaccharide and is composed of glucosamine and N-acetyl glucosamine units linked by β (1–4) glycosidic bonds (Chan, Kurisawa, Chung, & Yang, 2007). Chitosan and its derivatives have various biological activities including immunoenhancing effects, antimicrobial, wound healing activities (Amiji, 2004; Cho, Han, & Ko, 2000) and molecularly imprinting properties (Ostovan, Ghaedi, Arabi, Yang et al., 2018; Ostovan, Ghaedi, & Arabi, 2018).

Chitosan and its derivatives have recently attracted increasing attention in gene therapy because of their lower toxicity and immunogenicity than many of cationic polymers and lipids (Alves & Mano, 2008; Amiji, 2004; Jeevitha & Amarnath, 2013; Opanasopit et al., 2008; Park et al., 2013; Sajomsang et al., 2013; Zohuriaan-Mehr, 2005). Chitosan, which is protonated in acidic pH due to the presence of free amine groups in its structure, easily forms polyelectrolyte complex with negatively charged DNA. However, due to its low transfection efficiency, modification of chitosan at the molecular level has gained importance to improve its potential in gene delivery

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applications. In previous studies, various modifications on chitosan including nitration, sulphation, phosphorylation, acylation, hydroxyalkylation, thiolation, graft polymerization, amination have been carried out to improve its transfection efficiency (Saranya, Moorthi, Saravanan, Devi, & Selvamurugan, 2011).

The aim of this study was to develop a novel nanoparticulate gene delivery system based on amine-functionalized chitosan (MChi). For this purpose, MChi was first synthesized in house with the aim of improving the gene transfer ability and of reducing the toxicity of nanoparticles prepared. The nanoparticles of MChi (nMChi) were fabricated by ionic gelation method followed by intense optimization studies which included their particle size, size distribution and surface charge. Then, cytotoxicity and transfection efficiency of the nanoparticles on Human Embryonic Kidney (HEK293) and Primary Ovine Fibroblast (POF) cell lines were determined. Green Fluorescent Protein circular plasmid DNA (pEGFN1) loaded nanoparticles (gnMChi) were used in the transfection studies.

2. Material and methods

Low molecular weight Chitosan (Chi) (75–85% deacetylated), N-(2-hydroxyethyl)ethylenediamine (HE-EDA), sodium tripolyphosphate (TPP), tris base, EDTA, agarose, ethidium bromide (for the molecular biology) and bromphenol blue were purchased from Sigma-Aldrich (USA). Glacial acetic acid and acetone were obtained from Merck (Germany).

Dulbecco modified eagle medium (DMEM), Fetal calf serum (FCS), L-glutamate, sodium pyruvate, sodium bicarbonate, penicillin, streptomycin and trypan blue were obtained from Sigma (USA).

2.1. Synthesis of modified chitosan (MChi) and characterization

Chi was dissolved in 4% acetic acid, and then HE-EDA was poured into the Chi solution (0.5 mol HE-EDA per 1 mol glycosamine unit) and mixed vigorously at 70 °C for 30 min. MChi was precipitated in cold acetone, dried under vacuum at 40 °C and stored at + 4 °C. MChi was characterized using Fourier Transform Infrared (FTIR) spectroscopy technique (a sample/KBr ratio is of 1/200; Agilent Cary 630, USA) and proton nuclear magnetic resonance (^{1}H NMR) spectrophotometer (Varian UNITY INOVA, DMSO-d_6:D_2O, 500 MHz and 25 °C). The molecular weight of Chi and MChi was determined by GPC-SEC system (Tosoh Bioscience, Japan) (TSK-gel GMPWXL column, the solution at a flow rate 1 mL/min, dn/dc: 0.142 mL/g). Chi and MChi were dissolved in 1% acetic acid solution overnight then it was diluted to half with 0.2 mol/L sodium acetate solution, was passed through 0.22 µm membrane filter and transferred to the vials (ASTM F2602-08). The weight average molecular weight of Chi and MChi were 278.0, and 117.6 kDa and PDI (d = M_w/M_n) were also 1.961 and 1.261, respectively.

2.2. Preparation of nMChi

MChi was dissolved in 1% acetic acid (3 mg/mL) and diluted with distilled water to prepare at its dispersions at different concentrations (Table 1). nMChi were prepared by the addition of aqueous TPP solution (0.1% w/v) into MChi solution while magnetic stirring (250 rpm) at room temperature approximately one h. Formulation parameters were given in Table 1.

2.3. Characterization of nMChi

The mean particle size and size distribution of nMChi were determined by photon correlation spectroscopy (Nano ZS, Malvern, UK) (n = 6). Size of particles is expressed as Z-average diameter and reported as the mean diameter ± standard deviation (SD).

The surface charge of nMChi was determined by measuring zeta potential of particles using the electrophoretic light-scattering technique (Nano ZS, Malvern, UK). For each sample, three individual runs were performed for which zeta potential was calculated as the mean value SD (Table 2).

Table 1
The parameters for the preparation of the nMChi.

Nanoparticle	MChi (%)	MChi Solution (mL)	MChi:TPP Ratio (w/w)	TPP Solution (mL)
$nMChi_1$	0.05	3.33	3:1	3.3
$nMChi_2$			4:1	2.5
nMChi $_3$			5:1	2.0
nMChi $_4$	0.10	6.67	3:1	6.7
nMChi $_5$			4:1	5.0
nMChi $_6$			5:1	4.0
nMChi $_7$	0.15	10.00	3:1	10.0
nMChi $_8$			4:1	7.5
nMChi $_9$			5:1	6.0

Table 2
The zeta potential, particle size and PDI values of nMChi.

Nanoparticle	Particle Size (nm)	PDI[a]	Zeta potential (mV)
nMChi $_1$	125.2	0.497	29.8 ± 4.23
nMChi $_2$	104.4	0.402	43.9 ± 4.43
nMChi $_3$	132.0	0.753	40.9 ± 3.56
nMChi $_4$	108.9	0.405	32.2 ± 5.41
nMChi $_5$	102.9	0.404	41.9 ± 5.63
nMChi $_6$	118.7	0.420	5.31 ± 3.62
nMChi $_7$	104.8	0.366	28.8 ± 4.47
nMChi $_8$	114.8	0.326	43.7 ± 8.89
nMChi $_9$	203.9	0.302	35.9 ± 3.82

[a] The Chi selling in the market has the wide range molecular weight distribution, so almost all of the nanoparticles prepared from MChi have about 0.400 PDI value.

2.4. The preparation of nanoparticulate gene carrier systems (gnMChi) and gel electrophoresis analysis

In order to prepare pEGFN1 loaded nanoparticles, empty nanoparticles and pEGFN1 at different nanoparticle:DNA ratios (w/w) were incubated for 30 min at room temperature while stirring on magnetic stirrer at 200 rpm. pEGFN1 loading capacity on the surface of the nMChi, Chi nanoparticles (nChi) and DNA integrity was assessed by 4% agarose gel electrophoresis (Cleaver Scientific Ltd., England).

A series of pEGFN1 loaded nanoparticles at different nanoparticle:DNA ratios (w/w) was loaded into the wells by mixing with loading dye in a ratio of 9:1 v/v and electrophoresis was carried out at a constant voltage of 100 V for 1 h in Tris-Acetate-EDTA (TAE-1X) buffer containing 0.5 g/mL ethidium bromide. The pEGFN1 bands were then visualized under a UV transilluminator at a wavelength of 254 nm.

2.5. In vitro cell culture studies

HEK293 cell line was purchased from American Type Culture Collection (ATCC). POF cells were obtained from slaughtered sheep ear skin at Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination. The growth media used for both cell line was DMEM supplemented with 10% FCS, Na-pyruvate (0.110 g/L), L-glutamine (0.584 g/L) and 2 mL MEM non-essential amino acid solution (100x) (DMEM + FCS).

Cultures were maintained in 75 cm^2 culture flasks (Orange Scientific, Belgium) at 37^{0}C in a humidified atmosphere with 5% CO_2. Media was changed every two days. When the cell monolayer reached 80–90% confluence, the cells were passaged using trypsin-EDTA solution (0.25% trypsin–0.8 mM EDTA) after washing twice with phosphate

buffered saline (PBS). Cell viability was determined through staining with Trypan blue and cells were counted using hemocytometer. The suspended cells were seeded into 96-well plates at a density of 30,000 cells per well for cytotoxicity studies.

2.6. Cytotoxicity of the nanoparticles

In vitro cytotoxicity of the nanoparticles was determined in real time by the xCELLigence RTCA system (Roche, Germany). DMEM + FCS (100 µL) was added in each well of 96 well E-Plate, and the plate was kept at room temperature for 30 min. E-Plate was placed in the xCELLigence device, and background value was measured.

HEK293 and POF cells (30,000 cells/well) were seeded in each well and kept for 30 min at room temperature. E-Plate was placed into the device, kept one day at 37°C for cell proliferation, and then culture medium was removed. Blank nChi, $nMChi_5$ and PEI, at various concentrations in 200 µL DMEM were added into each well and allowed to contact with cells for four h. After incubation, the media containing debris and nanoparticles were removed, and 200 µL DMEM + FCS was added into each well, and cell proliferation was followed for 72 h. Statistical analysis of the cell index results was performed by applying the "Mann-Whitney U" test in the "SPSS 13.0 For Windows" statistical program. Experiments were repeated four times (n = 4).

2.7. Transfection studies

In vitro transfection studies were performed in HEK293 and POF cell lines by using pEGFN1 loaded nChi (gnChi) and pEGFN1 loaded nMChi (gnMChi). The cells (20.000 cells/well) were seeded in each well and incubated in 5% CO_2 at 37 °C for 24 h. When cell monolayer was observed to reach about 40–50% confluence, growth medium (DMEM + FCS) was removed and cells were gently rinsed twice with DMEM.

Then, DNA-loaded nMChi at different amounts and 150 µL DMEM were added onto the cells (Table 3) and incubated at 37 °C for 4 h. Afterwards, medium was removed and substituted with 400 µL DMEM + FCS. *In vitro* culture was continued for 72 h and then the transfection efficiency was analysed with Olympus IX71 reverse microscope under 460–480 nm fluorescence light. The transfection studies were also repeated with gnChi as a control group using the same procedure. The transfected cells were identified based on the green fluorescence glowing cells and the numerical values were obtained by examining the photographs of the specific regions on wells with the imaging system.

3. Results and discussion

3.1. Synthesis and characterization of MChi

The synthesis of MChi was carried out *via* the modification reaction between the HE-EDA and the Chi in acidic conditions to increase the amine content of the aliphatic pendant group on the chitosan backbone.

The modification reaction took place in the form of an elimination/condensation reaction mechanism (Cho et al., 2000). Acetic acid was used as a catalyst for the elimination of the hydroxyl group of the methylol groups. At the same time, acetic acid was used as a reagent to dissolve chitosan molecule by transformation from amine groups to ammonium salts so they can act as a nucleophile because of their positive charges. As seen in Fig. 1, the modification reaction was carried out between the hydroxyl groups of HE-EDA and the methylol groups of chitosan in acidic conditions.

The formation of MChi product was confirmed by FTIR and ^{1}H NMR spectral analysis. Fig. 2 shows the FTIR spectra of Chi and MChi. FTIR spectra of Chi was exhibited the characteristic absorption bands of the Chi molecule. The broad band at about 3750–3000 cm^{-1} region was related to the stretching vibrations of the primary NH_2 groups (max. at 3430 cm^{-1}) and the stretching vibrations of the OH groups (max. at 3394 cm^{-1}) in the chitosan molecule, the small broad band at 3000-2800 cm^{-1} region (max. at 2926 and 2877 cm^{-1}) was due to the symmetric and asymmetric stretching vibrations of CH_2 groups of the pyranose rings in chitosan molecule, the broad band at max. 1653 cm^{-1} was related to the stretching vibrations of the C═O groups of the amide I bands (in the acetyl-amide units), the broad shoulder at about 1594 cm^{-1} was due to the bending vibrations of the primary NH_2 groups, the small broad bands at about 1450–1250 cm^{-1} region (max. at 1426, 1377 and 1322 cm^{-1}) were due to the bending vibrations of mainly the free methylol groups and partially stretching vibrations of CH_3 groups in the acetyl groups, the broad band at 1200–900 cm^{-1} region (max. at about 1154 and 1075, 1032 cm^{-1}) was due to the symmetric and asymmetric stretching vibrations of C–O–C bonds, C–O bonds and skeletal vibrations of Chi molecule, respectively. The small band at 898 cm^{-1} was related to the stretching vibrations of the acetyl groups of the Chi molecule (Socrates, 2001).

The formation of the MChi product was connected with the decrease of the intensities or disappearance of the small and broad at about 1450-1300 cm^{-1} region (max. at 1426, 1377 and 1322 cm^{-1}) due to the bending vibrations of the free methylol groups ($-CH_2OH$) onto Chi molecule, notwithstanding the appearances of a new broad, sharp and small absorption bands at about 3433 (its intensity increased), 1639, 1566 and 1405 cm^{-1} due to the stretching vibrations of the primary NH_2 groups, the deformation vibrations of the NH bonds of the secondary amine structure in the HE-EDA molecule and the stretching vibrations of the $-CH_2OCH_2$- bonds in the aliphatic ether structure formed in the MChi, respectively (Wang & Liu, 2014).

The ^{1}H NMR spectra of the Chi and MChi were shown in Fig. 3. In the spectra related to Chi (Fig. 3A), the characteristic signals of the methylene ($-CH_2-$) protons and hydroxyl ($-OH$) protons in the molecular structure were observed at 0.854 ppm, 1.236 ppm and 1.467 ppm, respectively. Similarly, the signals of the hydroxyl ($-OH$) protons of the methylol ($-CH_2OH-$) groups were around at 1.897 ppm, 1.979 ppm, 2.164 ppm, 2.362 ppm, 2.636 ppm, and 5.323 ppm. The signals from $-CH_2-$, $-NH_2$ and $-CH-$ groups/bonds protons in the repeated units of the Chi molecular structure also resonated at 0.854 ppm, 1.897 ppm, 1.979 ppm, 2.087 ppm, 2.164 ppm, and 2.636 ppm, respectively (Guo, Xing, Liu, Zhong, & Li, 2008).

The signals of the hydroxyl ($-OH-$) protons of the $-CH_2OH$ groups (at 1.467 ppm, 1.979 ppm, and 2.164 ppm) were not detected in the ^{1}H NMR spectra of MChi (Fig. 3B), and the intensity of the signal at 1.234 ppm was also reduced. On the other hand, the new signals appearing at 1.146 ppm, 1.132 ppm, 1.899 ppm and 2.025 ppm, at 1.768 ppm, at around 2.6–2.9 ppm region (as the three split peaks) and at around 3.2–3.6 ppm region (max. at 3.457 ppm, 3.468 ppm and 3.479 ppm) (as multi peaks overlapping with the signals corresponding to the water in DMSO) are assigned to the methylene ($-CH_2-$) protons in the $-CH_2-CH_2-NH-CH_2$- units, the methylene ($-CH_2-$) protons in the $-CH_2-O-CH_2-CH_2$- units, the methylene ($-CH_2$-) protons in the $-CH_2-CH_2-NH-CH_2$- and $-CH_2-CH_2-NH-CH_2-CH_2-NH_2$ units, and the methylene ($-CH_2$-) protons in the $-CH_2-O-CH_2-CH_2$- units, respectively (Oyervides-Muñoz, Pollet, Ulrich, de Jesús Sosa-Santillán, & Avérous, 2017; Woraphatphadung, Sajomsang, Gonil, Saesoo, & Opanasopit, 2015).

Modification reaction mechanism which was proposed in Fig. 1 was consistent with FTIR and ^{1}H NMR spectra of the Chi and MChi. All these changes and the appearances of the new bands and signals in the spectra showed that the reactions between Chi and HE-EDA occurred according to the reactions presented in Fig. 1.

3.2. Characterization of nanoparticles

Physical characteristics such as particle size, size distribution, and surface charge are highly important for the efficiency of cellular uptake and subsequent intracellular processing of nanoparticles, which are

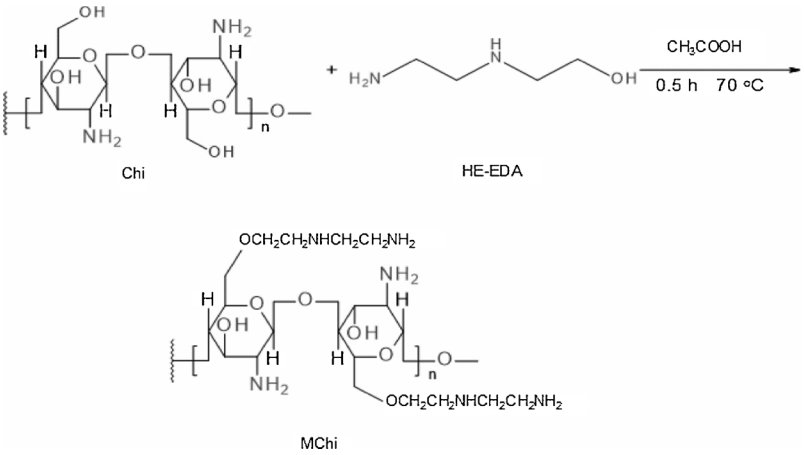

Fig. 1. Modification reaction between Chi and HE-EDA.

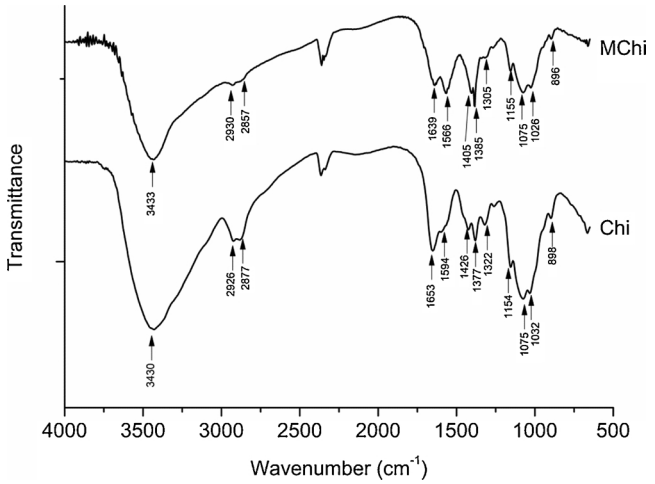

Fig. 2. FTIR Spectra of Chi and MChi.

prerequisite for effective cellular transfection. Despite the fact that it is generally difficult to determine the various factors arising from cells containing intercellular spaces, diffusion velocity in the spaces, and capillary endothelium penetration, the particles should be smaller than 200 nm preferably in the size range of 20–150 nm because liver efficiently capture and eliminate particles on the scale of 10–20 nm (Lavertu, Methot, Tran-Khanh, & Buschmann, 2006). The particles showed mean size between 102.9–203.9 nm with polydispersity index ranging from 0.302 to 0.753 and polymer:TPP mass ratio significantly influenced their particle size and size distribution.

Positive zeta potential, which plays an important role on complex formation of nanoparticles and pDNA, effective cellular uptake and transportation of DNA into the nucleus, should also be at high level. Zeta potential of the nanoparticles is related with neutralization of the amine groups or degree of deacetylation of polymer and should be usually +30 mV and above in the transfection studies (Schatzlein et al., 2005).

In this study, nMChi were prepared *via* the ionic gelation method (Table 1). nMChi resulted in a higher positive zeta potential value between +29.8 mV and +43.9 mV and polymer:TPP mass ratio did not significantly influence the surface charge of the microspheres.

As seen in Table 2, nMChi except $MChi_6$ formulation which has low zeta potential value are convenient as gene delivery system. Especially $nMChi_5$ formulation with the smallest particle size which has particle size of 102.9 nm, PDI of 0.404 and zeta potential of 41.9 ± 5.63 mV is the most convenient formulations and this nanoparticles were used in the following experiments.

3.3. The gel electrophoresis analysis of nChi and $nMChi_5$

Gel electrophoresis analysis was conducted to determine the gene binding capacity of the nChi and $nMChi_5$. The $nMChi_5$ complexed with all pEGFN1 at a low and equal ratio (nanoparticle : DNA, w/w) of 1:1 due to the increase the amine groups on the Chi molecule as a result of the modification reaction. However, in the case of unmodified nChi, this complex (gnChi) can only form at a ratio of 4:1(w/w) (Fig. 4). Similarly, according to the transfection studies in the literature, the nChi complexed with all pEGFN1 at least 3:1 or 4:1 (w/w) (Jin et al., 2017; Picola et al., 2016). So the nMChi which complex all pEGFN1 at least 1:1 (w/w) ratio, gives more advantage regarding cytotoxicity characteristics at transfection studies.

3.4. The cytotoxicity of nMChi

The cytotoxicity tests of nMChi were conducted with two types of cells (HEK293 and POF) by RTCA. RTCA gives the calculated percentage of cell viability based on time-dependent cell index values.

The nChi and PEI were used as negative and positive control group, respectively. The selected cytotoxicity concentration levels of the nChi, PEI, and nMChi, are specified according to transfection studies (7.5 μg/mL) and highly above of that levels are analyzed in order to determine the most toxic level (25 μg/mL, 50 μg/mL, 150 μg/mL).

In general, PEI as a positive control group reduced the viability of HEK293 by 50% at all concentration levels used in working conditions. However, the cell viability was 90% and 60% at a PEI concentration of 7.5 μg/mL and 25 μg/mL, respectively, at the end of the 72nd h. However, it should be noted that the cell viability at a concentration level of 7.5 μg/mL reduces to a range of 40–50% even at the 28th h (Fig. 5A_1). Similarly, the high toxicity of PEI is more clearly observed in POF cells. The cell viability values are observed to be dramatically low at 28th and 72nd h (Fig. 5A_2).

The nChi used as a negative control does not show any toxic effects on the HEK293 and POF cells (Fig. 5B_1-B_2). Cytotoxicity of nMChi on the HEK293 cells, when compared to nChi, no significant decrease in cell viability was observed at both 28 and 72 h at all concentrations below 150 μg/mL concentration (Fig. 5C_1). Moreover, based on the cytotoxicity results of nMChi on the POF cells, there is no toxic effect at all concentrations (Fig. 5C_2).

3.5. The transfection efficiency of gnChi and $gnMChi_5$

The transfection efficiency experiments of $gnMChi_5$ and gnChi (control group) were carried out using both HEK293 and POF cells. The results of the transfection efficiency of gnChi and $gnMChi_5$ are given in Table 3 and the microscope images showing the highest transfection of

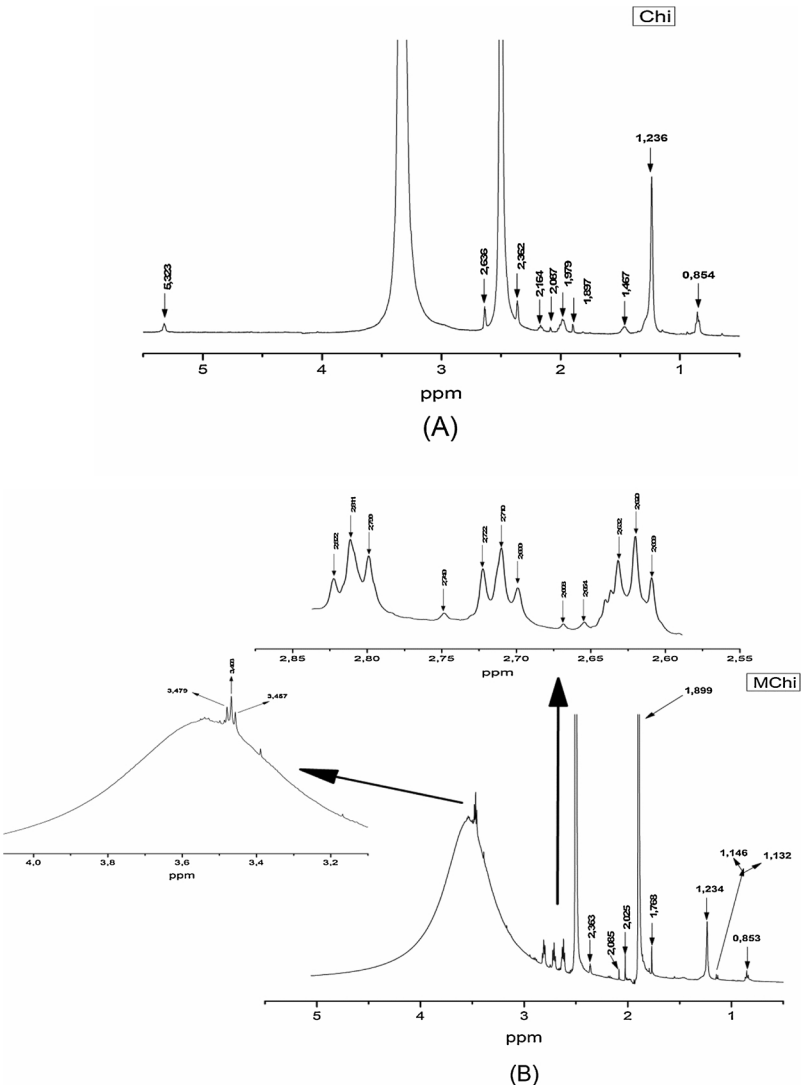

Fig. 3. ^{1}H NMR Spectra of A)Chi and B)MChi.

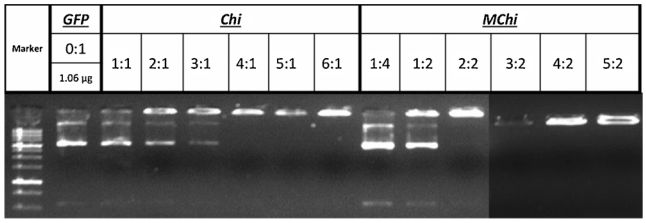

Fig. 4. Gene binding capacity of the nChi and nMChi$_5$ using Gel Electrophoresis analysis.

these nanoparticles are given in Fig. 6A–C. As seen in Table 3, the transfection efficiency of nChi was 15% in HEK293 cells. When the nChi: pEGFN1 ratio was used as 4:1 and 6:1 (w/w), the amount of plasmid was used as 2.12 µg, the results of the transfection were the highest value. On the other hand, no transfection activity was observed in the POF cells. In case of gnMChi$_5$, the highest transfection efficiency (95% for HEK293 and 20% for POF cells) were observed at gnMChi$_5$: pEGFN1 ratio of 7:1 (w/w) and the amount of plasmid of 2.12 µg.

As known, the transfection efficiency mainly depends on the method, the type of cells and the properties of nanoparticles used as a carrier. There are various methods for gene transfer into mammalian cells. However, the efficient gene transfer into most of the primary cells has not been resolved because of these methods do not allow efficient and safe transfer of DNA or RNA. Therefore, primary cells are separately evaluated in gene transfer studies (Gresch et al., 2004). Kim et al. (2007), also pointed out that the chitosan nanoparticles have low transfection efficiency in primary cells. This situation was also observed in our study; the gnMChi$_5$ had high efficiency in HEK293 cells (95%). Nevertheless, the transfection in the POF cells was very low (20%), but, acceptable for the POF cells.

The gene transfection process consists of DNA complexation, cellular uptake, escape from endosomes, dissociation of the DNA from the carriers, and DNA integration into the nuclear DNA (Mao, Ma, Jiang, et al., 2007; Mao, Ma, Yan, et al., 2007). Complexation between DNA and Chi strongly depends on the number of protonated amino groups, the number of interacting charges per molecule (valence) and distribution of charges along the chain (the charge density) of the Chi (Strand, Danielsen, Christensen, & Vårum, 2005). Chi, a native aminopolysaccharide, was a weak polyelectrolyte (p*K*a of 6.5–6.6) and water-insoluble due to its strong intermolecular and intramolecular hydrogen bonds. In acidic media (pH < 6.5), Chi becomes water soluble due to the protonation as a polycation (Strand et al., 2005). Chi-DNA complexes are formed between fully ionized anionic phosphates groups and the fraction of protonated chitosan by electrostatic interactions in the solution (Bravo-Anaya, Soltero, & Rinaudo, 2016). The changes in the pH of the solution can influence the degree of ionization of Chi, resulting in increased or decreased electrostatic interactions.

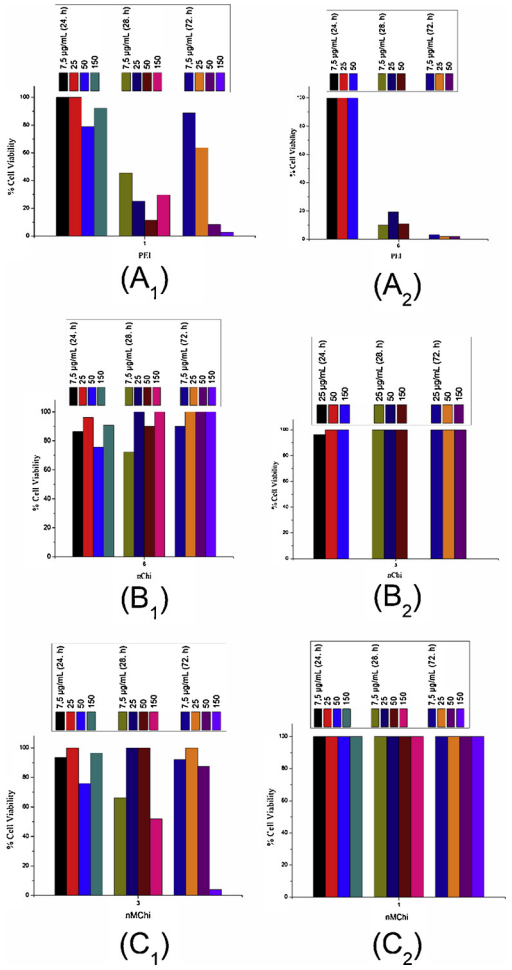

Fig. 5. The cytotoxicity results of (A_1) PEI, (B_1) nChi, (C_1) nMChi on the HEK293 and (A_2) PEI, (B_2) nChi, (C_2) nMChi on the POF cells.

According to the hypothesis of Kotze, Luessen, de Boer, Verhoef, and Junginger' (1999), to obtain an advantage in the applications of Chi as a drug or DNA carrier, an improvement of permanent positive charge on Chi backbone, to provide new functionalities and properties such as improving solubility, cell specificity, and the transfection efficiency as well as reducing cytotoxicity is needed. This can be achieved by covalently addition of a substituent containing amine groups as the pendant functional groups. Therefore, modified chitosan vectors with higher positive charge strength have a stronger ability to combine with DNA.

Based on the data of the transfection studies realized in this study, $gnMChi_5$ exhibits a higher transfection efficiency than nChi for both cell types. The gene transfer capacity of nChi increased by the modification with aliphatic amine compound, HE-EDA. Besides, as a result of gel electrophoresis, nMChi combined with the same amount of pEGFN1 at a low and equal nanoparticle: DNA ratio of 1:1 (w/w) than that of nChi (4:1). nMChi contains long aliphatic pendant chains with amine end group (Fig. 1). These flexible linear side-chains greatly reduced the formation of intermolecular and intramolecular hydrogen bonds of the rigid Chi backbone. Amine end groups on the side-chains also enhanced both the positive charge density and the total valence of nChi, which have great importance in the complexation process. It was concluded that functionalization of Chi with amine compounds significantly lead to change its transfection efficiency, complexing ability with DNA and cytotoxicity. These results are also compatible with the hypothesis which suggested in literature (Kotze et al., 1999).

4. Conclusion

This study introduces the synthesis and the characterization of the long linear aliphatic amine-functionalized Chi (MChi) and the preparation of the nanoparticles nMChi for using in the transfection studies in both of two cell types, as HEK293 and POF cell lines, for the first time. When the Chi contains amine groups as long linear pendant group in the molecular structure, its transfection efficiency and complexing ability with DNA significantly increased. It was determined that the nMChi could make a complex with the same amount of DNA at four times less amount than the nChi. $gnMChi_5$ has the higher transfection

Table 3
Transfection efficiency results of gnChi and $gnMchi_5$.

Particle	Ratio (w/w)		Amount (μg)		Volume (μL)			Cells	Seeded Cells	Toxicity	Efficiency (%)
	Polymer	Gene	Polymer	Gene	Polymer	Gene	Total				
gnChi	2	1	4	2.12	4	4	8	HEK293	20000	Non-toxic	5
	3	1	6	2.12	6	4	10			Non-toxic	7
	4	1	8	2.12	8	4	12			Low-toxic	15
	5	1	10	2.12	10	4	14			Moderate-toxic	10
	6	1	12	2.12	12	4	16			Moderate-toxic	15
	7	1	14	2.12	14	4	18			Moderate-toxic	13
	2	1	4	2.12	4	4	8	POF9	20000	Non-toxic	0
	3	1	6	2.12	6	4	10			Non-toxic	0
	4	1	8	2.12	8	4	12			Non-toxic	0
	5	1	10	2.12	10	4	14			Non-toxic	0
	6	1	12	2.12	12	4	16			Non-toxic	0
	7	1	14	2.12	14	4	18			Non-toxic	0
$gnMChi_5$	2	1	4	2.12	4	4	8	POF 9	20000	Non-toxic	0
	3	1	6	2.12	6	4	10			Non-toxic	0
	4	1	8	2.12	8	4	12			Non-toxic	0
	5	1	10	2.12	10	4	14			Non-toxic	1
	6	1	12	2.12	12	4	16			Non-toxic	5
	7	1	14	2.12	14	4	18			Non-toxic	20
	2	1	4	2.12	4	4	8	HEK293	20000	Non-toxic	3
	3	1	6	2.12	6	4	10			Non-toxic	8
	4	1	8	2.12	8	4	12			Non-toxic	15
	5	1	10	2.12	10	4	14			Non-toxic	35
	6	1	12	2.12	12	4	16			Non-toxic	75
	7	1	14	2.12	14	4	18			Non-toxic	95

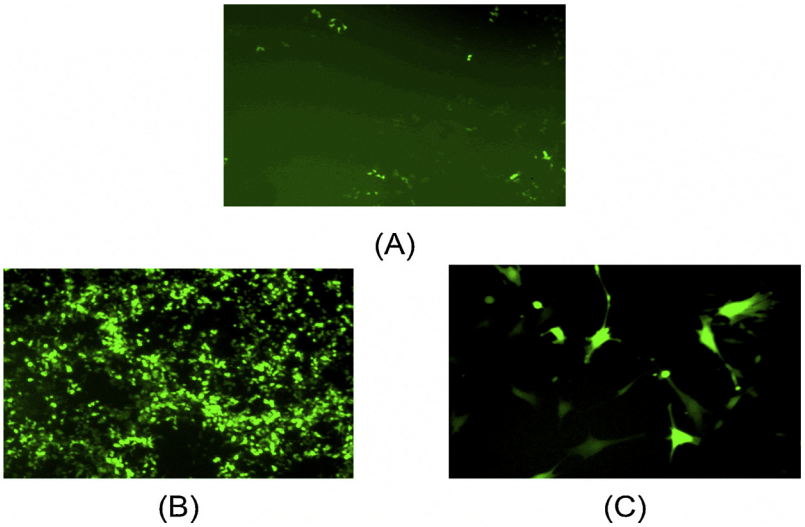

Fig. 6. The images of the transfected cells; (A) nChi: pEGFN1 ratio 6:1 (w/w) onto the HEK293 cells (x40), (B) nMChi: pEGFN1 ratio 7:1 (w/w) onto the HEK293 cells (x40), (C) nMChi: pEGFN1 ratio 7:1 (w/w) onto the POF cells (x200).

efficiency than nChi for both cell types. This is mainly attributed to an increase in the permanent positive charge density and the total valence, also to obstruction the formation of intermolecular and intramolecular hydrogen bonds of Chi backbone. While the chitosan nanoparticles have no transfection efficiency on primary cells, the efficiency was an acceptable for the amine functionalized chitosan nanoparticles. Based on the data of this study, it was concluded that gnMChi$_5$ with high transfection efficiency could also be used as the non-toxic nonviral gene delivery system on HEK293 and POF cell lines. It was observed that the amine functionalization of the chitosan fortified the applications of chitosan nanoparticles as novel nontoxic non-viral gene delivery systems with improved transfection efficiency on HEK293 and POF cells.

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