

ORIGINAL ARTICLE

Ultrafine chitosan nanoparticles as an efficient nucleic acid delivery system targeting neuronal cells

Meenakshi Malhotra, Arun Kulamarva, Safaa Sebak, Arghya Paul, Jasmine Bhathena, Maryam Mirzaei and Satya Prakash

Biomedical Technology and Cell Therapy Research Laboratory, Departments of Biomedical Engineering and Physiology, Artificial Cells and Organs Research Center, Faculty of Medicine, McGill University, Montreal, Quebec, Canada

Abstract

Background: Cell transfection with nanoscaled cationic polymeric particles using Chitosan has been extensively explored. Because of its properties such as cationic charges, biocompatibility, biodegradability, and low toxicity, it has been used as a potential gene, siRNA, protein (including antibodies), and drug carrier system. **Method:** This work describes the development of chitosan nanoparticles of a 20-nm diameter for a potential siRNA delivery application. The particles were prepared using an ionic gelation method, using sodium tripolyphosphate as a cross-linker. The effect of variation in pH was investigated on particle size and surface charge. Gene loading efficiency by chitosan nanoparticles was performed by varying weight ratios of chitosan:siRNA. Transfection efficiency was evaluated on Neuro2a cells. **Results:** It was observed that 20-nm-sized nanoscale complexes induced significant transfection in neuronal cells. **Conclusion:** These particles have potential in the delivery of siRNA to neural tissues.

Key words: Delivery; neuronal cells; siRNA; transfection; ultra-small nanoparticles

Introduction

The efficacy of many therapeutic molecules is often limited due to the potential physiological and anatomical barriers in reaching the target site of therapeutic action. Crossing the blood–brain barrier (BBB) is a major challenge in delivering drugs for the treatment of neurodegenerative diseases¹. Hence, developing a delivery system that optimizes the pharmaceutical action of the drug, reduces the toxicity in vivo, overcomes the barriers, and delivers the drug at the targeted site is an exigent task². Several methods have been developed in the last decade to enhance the target-specific delivery of therapeutic molecules. The use of viral vectors has been extensively employed in this approach but is limited because of their oncogenic effects and nonspecific immunological reactions^{3,4}. This article focuses on development of nonviral, ultrafine nanoparticles made of chitosan as a delivery

system to pass through the BBB and deliver siRNA into neural cells for therapeutic purposes.

Chitosan and its derivatives have shown great potential in the areas of biotechnology, biomedicine, food ingredients, and cosmetics. Chitosan is a natural biocompatible polymer obtained from chitin by exhaustive deacetylation under high temperature and alkaline conditions. It has a unique chemical structure as a linear polyelectrolyte with a high charge density as well as reactive hydroxyl (OH) and amino groups (NH₂). This renders chitosan as an ideal delivery candidate for the delivery of oligonucleotides^{6,7}. Chitosan has been extensively studied because of its ideal properties including bioadhesiveness⁸, biocompatibility⁹, bioactivity, and biodegradability¹⁰.

Furthermore, chitosan can be easily engineered to develop micro- and nanoparticles which can be subsequently targeted to the type of targeted tissue. At the same time the drug dose, stability, circulation time, and

Address for correspondence: Professor Satya Prakash, MSc, MTech, PhD, DSc, Biomedical Technology and Cell Therapy Research Laboratory, Departments of Biomedical Engineering and Physiology, Artificial Cells and Organs Research Center, Faculty of Medicine, McGill University, 3775 University Street, Montreal, Quebec, H3A 2B4, Canada. E-mail: satya.prakash@mcgill.ca

(Received 11 Jun 2008; accepted 2 Oct 2008)

ISSN 0363-9045 print/ISSN 1520-5762 online © Informa UK, Ltd.
DOI: 10.1080/03639040802526789

<http://www.informapharmascience.com/ddi>

the in vivo environment of the cell can be optimized. Because of its cationic charges, biocompatibility, and low toxicity¹¹, chitosan has been used as a vehicle system for genes, protein (including antibodies), and drugs^{12–14}. The size, shape, and surface charge of chitosan-based micro- or nanoparticles are influenced by the degree of deacetylation, molecular weight, and pH of the chitosan solution^{15–17}. The entrapment efficiency depends on the pK_a , solubility of the molecule to be incorporated, the strength of electrostatic interactions, hydrogen bonds, and hydrophobic interactions between the molecule and chitosan¹⁸. Chitosan/DNA complexes especially from derivatized-chitosan have been reported to effectively transfect various cell types like THP-1¹⁹, HEK 293, CHO K1²⁰, HeLa^{21,22}, Caco-2, Cos-1¹³, HepG2²³, and NIH 3T3²⁴.

The mechanism of cellular uptake is facilitated by the presence of positively charged free amino groups ($-NH_2$) in chitosan, which become protonated on encountering an acidic pH in the endosome and form cationic amine groups ($-NH_3^+$), thereby leading to endosomal disruption^{25,26}. Various preparation techniques can be used to prepare chitosan nanocarriers by altering parameters such as concentration of the chitosan or the crosslinker, the molecular weight of chitosan ratio of drug:polymer, pH, and finally stabilizers or surfactants. All these factors collectively affect the structural and morphological properties of chitosan nanoparticles and the release rate of the loaded therapeutic molecule²⁷. This article describes a method for the production of ultrafine nanoparticles and investigates the impact of various formulation and preparation parameters. The targeted delivery of siRNA using chitosan nanoparticles to the neural cells in vitro has been evaluated.

Materials and methodology

Chemicals and reagents

Low-molecular weight (LMW), medium-molecular weight (MMW), and high-molecular weight (HMW) chitosan were obtained from Wako (Richmond, VA, USA), each having a viscosity of 5–20 cP and degree of deacetylation of 80.0%. Sodium triphosphate pentabasic (TPP) and glacial acetic acid were obtained from Sigma (Oakville, ON, Canada). For dilution purposes ultrapure double-distilled water (ddH_2O) was used from laboratory-installed Barnstead Nanopure diamondTM water supply unit. siGLO (Green) transfection indicator (20 nm) was obtained from Dharmacon (Lafayette, IN, USA). Agarose (low gelling temperature) for gel electrophoresis was obtained from Sigma. CellTiter 96[®] Aqueous One Solution Reagent was purchased from Promega (Madison, WI, USA) to perform cell viability

assay, ethidium bromide (10 mg/mL) for band visualization on electrophoretic gels was obtained from Sigma. Orange dye solution (6X) was obtained from Fermentas (Burlington, ON, Canada) and TrackIt 10-bp DNA ladder (0.5 $\mu\text{g}/\mu\text{L}$) was obtained from Invitrogen (Burlington, ON, Canada). Eagle's minimum essential medium (EMEM) was obtained from ATCC and supplemented with 10% fetal bovine serum (FBS).

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by an ionic gelation procedure as explained in previous studies²⁸. Briefly, chitosan of different molecular weight were dissolved in 1% acetic acid solution to yield a concentration of 0.5 mg/mL at pH 5. TPP was dissolved in ddH_2O to obtain a concentration of 0.7 mg/mL and the pH was adjusted to 3 and 9. Nanoparticles were formed after addition of TPP (dropwise) to chitosan solution under constant magnetic stirring for an hour at room temperature. Different weight ratios of chitosan-TPP starting from 3:1 to 7:1 were used in a systemic study to yield the minimum possible size of nanoparticles (<100 nm).

Preparation of siGLO-entrapped chitosan nanoparticles

For siGLO to be entrapped into the chitosan nanoparticles, siGLO (266 $\mu\text{g}/\text{mL}$) was mixed with TPP, before adding it dropwise to the chitosan solution. The complete solution was kept under magnetic stirring for an hour at room temperature. The siGLO was mixed at varying weight ratios (1:100 to 1:250) with chitosan-TPP nanoparticles to achieve maximum gene loading into the nanoparticles.

Characterization of chitosan-TPP nanoparticles or chitosan-TPP/siGLO nanoparticles

Nanoparticles were characterized for mean particle diameter and size distribution using Brookhaven BI-90 Particle Nanosizer. Nanoparticle surface charge (zeta potential) was determined using Malvern Zeta sizer at 25°C.

Morphology

The nanoparticles were observed under high-resolution transmission electron microscope (TEM) (JEOL JEM 2000FX Electron Microscope). A drop of the sample containing the nanoparticles was placed on a carbon film-based copper mesh grid and allowed to air dry before observation.

siGLO-loading efficiency

The loading efficiency of siGLO to chitosan polymer was determined by gel electrophoresis of 48 μL of the sample

on a 4% (w/v) agarose gel. Samples with chitosan-TPP:siGLO at a weight ratio of 100:1, 150:1, 200:1, and 250:1, along with 1:6 dilution of the 6× orange dye was loaded onto the gel and was run for 4 hours at 55 V in Tris-borate EDTA (TBE) buffer (pH 8.3). The TBE buffer contained ethidium bromide at a concentration of 0.5 µg/mL, which is required for the visualization of RNA bands under UV transillumination at 365-nm.

Transfection efficiency

Transfection studies were performed on mouse neuroblastoma cells (Neuro2a). The cells after reaching a confluency of approximately 85% were subcultured and seeded in a 96-well plate at a density of 20,000 cells per well with 200 µL of complete growth media (antibiotics-free EMEM with 10% serum). After 24 hours, transfection media containing 100 µL of serum-free medium along with 50 µL of sample was added onto the cells. The tested samples were 50 µL of sample containing chitosan-TPP nanoparticles as positive control, free siGLO as negative control, and chitosan-TPP/siGLO nanoparticles at ascending weight ratios of 100:1, 150:1, 200:1, and 250:1 as treatment samples. The cells were then incubated at 37°C, 5% CO₂ for 4 hours, after which cells were observed under a fluorescence microscope (Nikon Eclipse TE2000-U) at a wavelength of 490 nm. siGLO is a transfection indicator gene; to fluorescence inside the cell enables the cells to be visualized under fluorescent microscope. Quantitative measurement of transfection was done using a microtiter plate reader (Perkin Elmer® 1420 Multilabel Counter Victor 3TM V) at 490 nm.

Results and discussion

Particle size and surface charge

Chitosan of different molecular weights LMW (10 K), MMW (100 K), and HMW (500 K) were crosslinked with TPP at varying pH conditions. While maintaining the same physiological conditions such as pH, temperature and concentration, the chitosan nanoparticles showed a continuous trend of increasing size as the molecular weight increased (Figure 1). In our attempts to reach the smallest possible particle size, different weight ratios of chitosan: TPP starting from 3:1 to 7:1 were tested. The smallest obtained particle size was approximately 76 ± 1 nm with LMW chitosan (solution pH value = 5) and TPP (solution pH value = 3) with a positive surface charge of 16.54 ± 1.64 mV at a weight ratio of 3:1 (as illustrated in Figures 2 and 3). Indeed, TPP being an anionic reagent acts as a crosslinker and binds to the cationic chitosan because of electrostatic attraction²⁹. While the

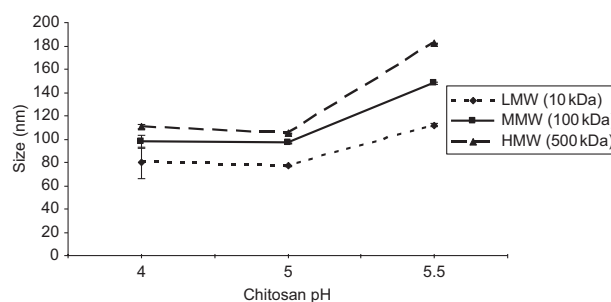


Figure 1. The influence of chitosan molecular weight on the nanoparticle size at three different pH values of chitosan solution. High-molecular weight (HMW), medium-molecular weight (MMW), and low-molecular weight (LMW) chitosan.

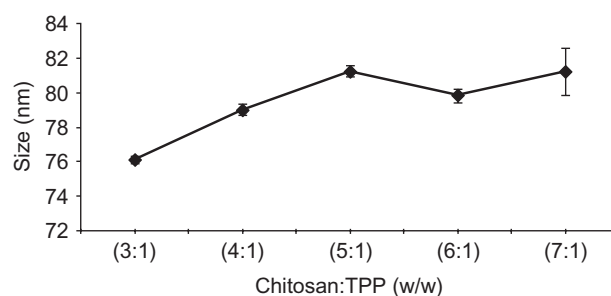


Figure 2. Effect of different weight ratios of low-molecular weight (LMW) chitosan (pH 5) crosslinked with TPP (pH 3) on nanoparticle size.

concentration of chitosan increases progressively from 3:1 to 6:1, with TPP at a constant concentration, the positive zeta potential/surface charge on the nanoparticles increases (with an exception at 7:1, at which zeta potential drops down again to 14.97 ± 1.57 mV) as shown in Figure 3.

Each particle sample was prepared in triplicates and assayed thrice on the Brookhaven BI-90 Particle Nano-

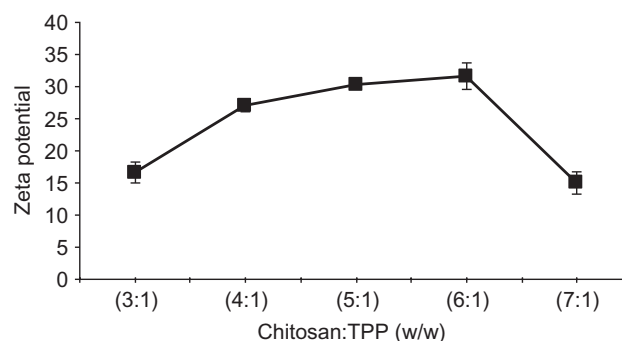


Figure 3. Effect of different weight ratios of low-molecular weight (LMW) chitosan (pH 5) crosslinked with TPP (pH 3) on zeta potential.

size for particle sizing and Malvern Zeta sizer for zeta potential. The results obtained were highly redundant and highly reproducible. The distribution of nanoparticles is mainly expressed by the polydispersity index which shows the percentage variation in the particle size of a sample. The polydispersity obtained was less than 0.4 in each case (data not shown), which confirms the sample as highly monodispersed. The particle size and their surface charge has been shown to be dependent on variable parameters like pH conditions and concentration. The particle size and surface charge were also affected by varying the molecular weight of chitosan and the different weight ratios of chitosan to TPP in the formulation³⁰.

Effect of pH on nanoparticle size

Results show that the chitosan solution at pH 5, yielded a smaller particle size, irrespective of the molecular weight of chitosan. The LMW chitosan yielded the lowest particle size of 76 ± 1 nm. The effect of pH of the TPP solution on particle size was investigated. TPP at pH 3 and 9 was allowed to crosslink with chitosan solution at pH 5. Various molecular weights of chitosan (10, 100, and 500 kDa) were tested to determine the effect of pH change in TPP. Results (Figure 4) show that the chitosan nanoparticles formed at TPP at pH 3 were smaller in size compared to particles formed with TPP at pH 9. This may be because TPP at an acidic pH condition has a lower (negative) charge density, which becomes a limiting factor. It is known that TPP at acidic pH interacts with a lesser number of positively charged chitosan units¹⁹. This results in the formation of monodispersed nanoparticles of smaller size as a lesser number of chitosan units are involved in the formation of the nanoparticles. TPP at basic pH bears a more negative charge and is

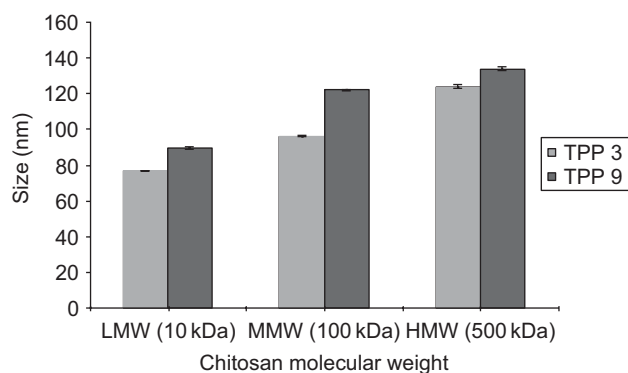


Figure 4. Effect of change of pH of TPP (pH 3 and 9) on the formation of chitosan-TPP nanoparticles made from HMW, MMW, and LMW chitosan.

thus able to interact with higher number of counter charges available on chitosan, yielding larger particle size (Figure 4). pH of both TPP and chitosan was found to play a significant role in nanoparticle size.

Transmission electron microscopy study

Nanoparticles were analyzed under TEM to study their morphological characteristics. Chitosan-TPP nanoparticles (without siGLO) were formed at chitosan solution pH 5 and TPP solution pH 3 and 9 at a weight ratio of 3:1. Chitosan-TPP nanoparticles (with entrapped siGLO) were formed at different weight ratios, ranging from 50:1 to 250:1 (chitosan-TPP: siGLO). On observation of siGLO-free nanoparticles, it was found that nanoparticles were irregular in shape and smaller in size (ranging between 50 and 70 nm, at TPP pH 3) compared to nanoparticles formed with TPP solution at pH 9 (greater than 200 nm in size) as shown in Figure 5b and c. siGLO-loaded/entrapped nanoparticles consisting of varying weight ratios from 50:1 to 150:1 of chitosan-TPP:siGLO were approximately in the range of 100–170 nm in size (Figure 5d–f). But at higher weight ratios of 200:1 and 250:1, the particles were monodispersed and effectively smaller (about 25 ± 5 and 50 ± 10 nm, respectively) as shown in Figure 5g and h. Interestingly, at these higher weight ratios, the smaller particle size was accompanied by a better entrapment efficiency of siGLO (Figure 6), which is probably due to the stabilization of the surface charge on the nanoparticles. This may also facilitate the cellular uptake of the nanoparticles^{14,31}. Our results were in accordance with a recent study that showed that complete entrapment of siRNA depends on the molecular weight of chitosan and weight ratio of chitosan-TPP with siRNA³⁰.

At weight ratio 50:1 (chitosan-TPP:siGLO), the nanoparticles appeared bigger and seemed to be aggregated. This could be because of the presence of free gene (siGLO) in the solution; being negatively charged it tends to attract the available positively charged chitosan units present in its vicinity. The unstable surface charge and the steric hindrance could lead to aggregation of nanoparticles. Our results are in accordance with reports in literature indicating that particle size decreases with decrease in molecular weight of chitosan but increases with increase in the concentration of the gene⁶. The TEM micrographs presented in Figure 5 give an idea of the size and shape of the nanoparticles, attributes which play a major role in the transfection of nanoparticles across the cellular membrane. Our results show that the lowest particle size of approximately 20 nm showed maximum transfection efficiency, whereas, as the size of the particles increased, the transfection efficiency became low.

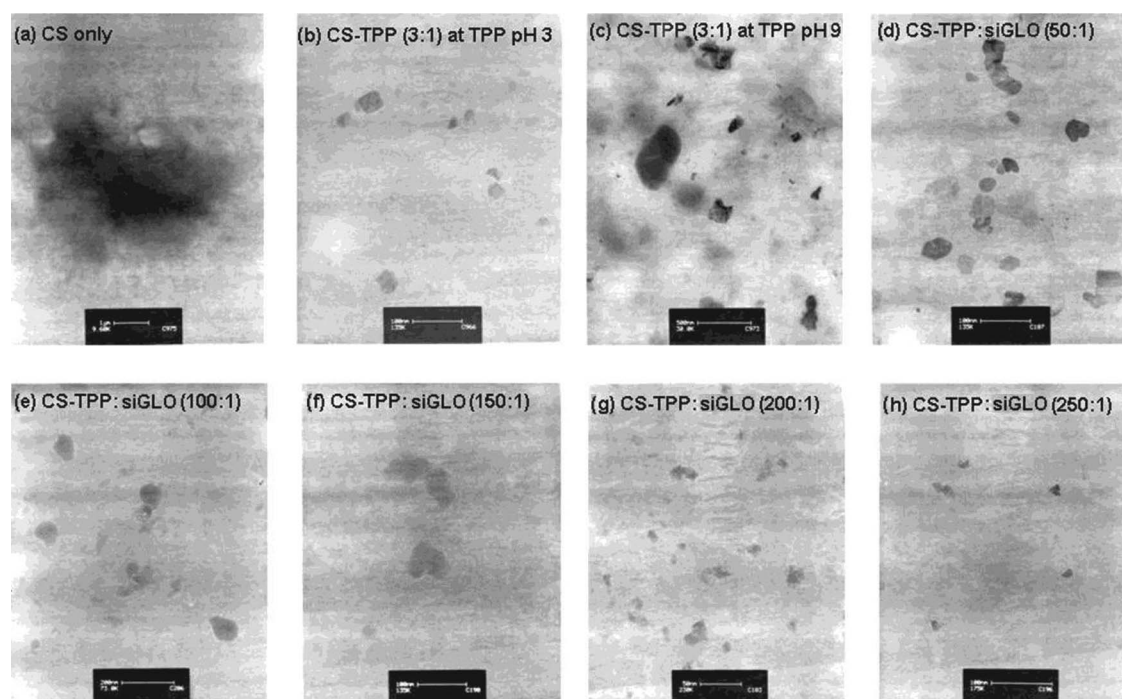


Figure 5. TEM micrographs of (a) chitosan polymer dissolved at pH 5, (b) Nanoparticles formed from complexation of chitosan and TPP with TPP at pH 3, (c) and TPP at pH 9, (d) siGLO-loaded chitosan-TPP nanoparticles with chitosan-TPP:gene weight ratio of 50:1, (e) 100:1, (f) 150:1, (g) 200:1, and (h) 250:1.

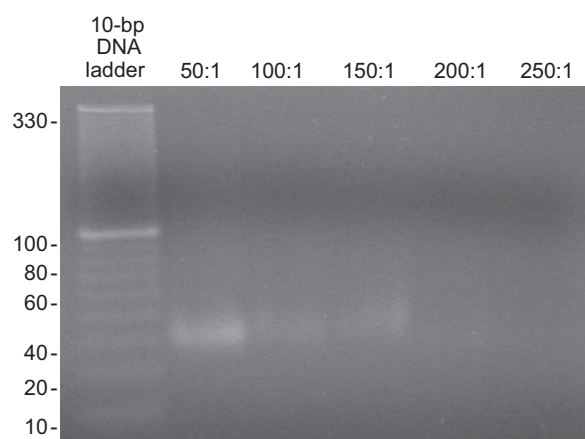


Figure 6. Agarose gel electrophoresis: Gel retardation assay of different weight ratios of chitosan-TPP with siGLO. Un-encapsulated siGLO in the suspension is observed in the 4% agarose gel. At 200:1 weight ratio of chitosan-TPP:siGLO, we achieved complete encapsulation of the gene, siGLO.

Gene-loading efficiency

We performed gel electrophoresis to evaluate the encapsulation of siGLO into the chitosan-TPP nanoparticles. In this agarose gel electrophoresis study, nanoparticles formed at different weight ratios were loaded into the

4% low gelling temperature agarose gel. 10-bp DNA ladder was used as a reference. The gel when observed under UV transilluminator, shows bands indicating the presence of unbound siGLO in the suspension as shown in Figure 6. As the concentration of chitosan in the chitosan-TPP:siGLO complex increased from 100:1 to 250:1, there was a gradual decrease in the intensity of bands. The change in intensity of the bands due to the increase in weight ratio and consequently of the strong interaction of chitosan with the gene, siGLO. The strong interaction relates to the greater number of available positive charges on chitosan that interact with negatively charged siGLO and TPP. This study proves the complete complexation of siGLO into the nanoparticles at weight ratio of 200:1 and 250:1. The amount of siGLO loaded at the weight ratio of 200:1 was 6 µg/mL of chitosan-TPP (w/w) solution.

Gene delivery and transfection efficiency

Transfection was performed with different weight ratios of chitosan-TPP:siGLO nanoparticles on Neuro2a, mouse neuroblastoma cells. Transfection efficiency was analyzed by measuring the fluorescence intensity of the siGLO-loaded nanoparticles. siGLO is a transfection indicator, which is a fluorescent oligonucleotide that is localized into the nucleus of the mammalian cell (Figure 7). The transfected cells were observed using a fluorescein

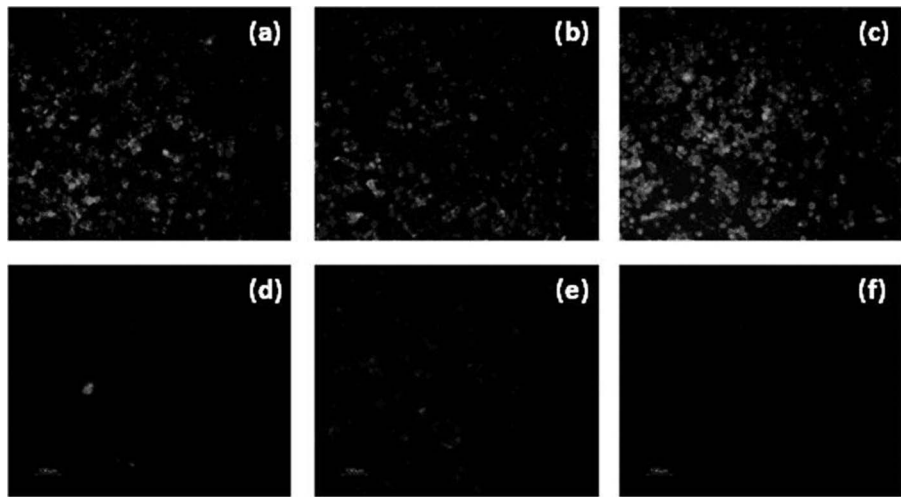


Figure 7. Evaluation of gene delivery and transfection efficiency: Neuro2a cells transfected with different ratios of chitosan-TPP:siGLO, (a) 100:1, (b) 150:1, (c) 200:1, (d) 250:1, (e) only siGLO, and (f) only cells (control).

isothiocyanate filter. siGLO, chitosan solution, and regular cells without any treatment were used as controls. Maximum transfection efficiency was observed when chitosan-TPP:siGLO nanoparticles at a weight ratio of 200:1 were used (Figures 7 and 8). A negligible amount of transfection was obtained with only siGLO. This confirms the results in a recent study, which show that Chitosan-TPP nanoparticles, as a cationic polymer formulation are an effective delivery mechanism even in comparison to lipophilic formulations and that they facilitate the uptake of genes by cells³². Being cationic, chitosan complexes are readily taken up by cells via a mechanism of endocytosis³³. In contrast, siGLO is negatively charged and is repelled by the negatively charged lipid-bilayer membrane of cells. Fluorescence intensity, as an indication of the transfection efficiency, indicates the importance of particle size and surface charge on

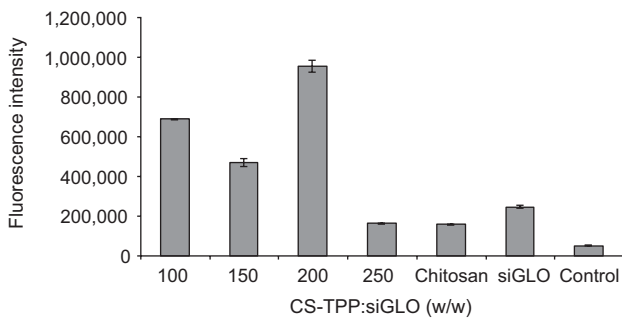


Figure 8. Transfection efficiency of siGLO-entrapped chitosan-TPP nanoparticles at different weight ratios starting from 100:1 to 250:1 (chitosan-TPP:siGLO) in Neuro2a mouse neuroblastoma cells. For CS-TPP weight ratio of 100:1, 150:1, 200:1, 250:1, the size of the nanoparticles were 110 ± 10 , 150 ± 10 , 25 ± 5 , and 50 ± 10 nm, respectively. siGLO, chitosan, and regular cells were used as controls.

nanoparticle uptake^{34,35}. The particle formed with 200:1 (w/w) chitosan-TPP:siGLO exhibits maximum uptake by the cells (Figures 7c and 8). The relation between weight ratios of chitosan-TPP:siGLO nanoparticles, particle size, and the transfection efficiency is more comprehensively expressed in Table 1. These results were in accordance with a previous study that showed LMW chitosan is an efficient nucleic acid-delivery system²⁸.

Like PEI, chitosan is known to exhibit a proton sponge effect which is highly pH dependent³⁶. At acidic pH, the chitosan attracts positively charged H^+ ions and gets protonated. The protonated positively charged chitosan attracts negatively charged Cl^- ions toward it. Therefore, an accumulation of H^+ and Cl^- ions occurs within

Table 1. This table explains the transfection efficiency obtained in Figures 7 (qualitative data) and 8 (quantitative data).

Sample	Chitosan-TPP: siGLO (w/w)	Particle size (nm)	Transfection efficiency (X: fluorescence intensity)
(a)	100:1	110 ± 10	XXX
(b)	150:1	150 ± 10	XX
(c)	200:1	25 ± 5	XXXX
(d)	250:1	50 ± 10	X

The transfection efficiency of chitosan-TPP:siGLO nanoparticles in Neuro2a cells was observed to be highly dependent on the particle size. The lowest particle size obtained of ~ 20 nm at weight ratio 200:1 showed highest transfection efficiency; also, the siGLO was completely encapsulated at this ratio. It was observed that as the particle size increased the transfection efficiency decreased gradually with an exception at a weight ratio of 250:1. It is anticipated that at the ratio 250:1 the siGLO amount as compared with the chitosan-TPP was too low to yield sufficient fluorescence. Weight ratios 100:1 and 150:1 lacked complete encapsulation of siGLO as shown in Figure 6 (gel electrophoresis data), and the particle size of more than 100 nm were anticipated to be the cause of low transfection efficiency in Neuro2a cells.

the cell's cytoplasm in the vicinity of the chitosan. This leads to an osmotic pressure difference between the exterior and the interior of the cell. Thus, the cell as well as the chitosan starts to absorb water and swell. The swelling helps the chitosan to escape the endosomal cavity and reach the cytoplasm³⁷. Moreover, it also helps entrapped genes to evade enzymatic attack by nucleases in endosomes, lysosomes, and follow the path toward nuclear import^{35,37,38}. The transfection indicator siGLO used in our experiment was localized inside the nucleus, therefore, it is quite apparent that the chitosan-TPP nanoparticles were easily released from the endosomal compartment and that the gene was imported to the nucleus effectively. As explained in previous studies^{15,39}, the pH of the medium and the molecular weight of the chitosan both play a key role in the transfection of nanoparticles. In a recent study, it was shown that the pH change of TPP from alkaline to acidic leads to controlled release of the oligonucleotides⁴⁰. Also apart from being an efficient crosslinker TPP being negatively charged, enhances the release of the gene, as it competes for the available positively charged groups on chitosan inside the cell.

Conclusion

Chitosan-TPP nanoparticles are an efficient delivery system for oligonucleotides ranging from 18 to 21 bp (siRNAs) into neuronal cells. In our study we developed ultrafine, monodispersed, siRNA encapsulated chitosan-TPP nanoparticles of 20 nm size formed by ionic gelation. Furthermore, our LMW chitosan nanoparticles exhibited excellent transfection efficiency as an indication of biological activity in Neuro2a mouse neuroblastoma cells. This delivery system needs to be further optimized using suitable surface coating molecules and ligands in order to represent a novel promising tool for gene delivery systems. Biocompatibility studies are needed to evaluate the suitability of these nanoparticles for in vivo use. The use of siGLO as a transfection agent is convenient and could prove to be superior to GFP and luciferase transfection agents to efficient siRNA delivery.

Acknowledgment

We gratefully acknowledge the assistance received from the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada (NSERC) to Dr. S. Prakash. The Centre of Biorecognition and Biosensors (CBB) for their support. We also acknowledge the support of the McGill Faculty

of Medicine Internal Scholarship to M. Malhotra. A.K. acknowledges the Alexander Graham Bell Post Graduate Scholarship—Doctoral from NSERC. J.B. acknowledges a Doctoral Scholarship from CIHR. The authors acknowledge the technical support provided by Dr. Misara Hamoudeh. S.S. acknowledges the McGill Faculty of Medicine Internal Scholarship. We are grateful to Prof. Maryam Tabrizian for providing us with facilities (Malvern Particle Sizer and Brookhaven Zeta Potential) in her Laboratory at McGill University, Canada.

Declaration of interest: The authors report no conflicts of interest.

References

- Sanovich E, Bartus RT, Friden PM, et al. (1995). Pathway across blood-brain barrier opened by the bradykinin agonist, RMP-7. *Brain Res*, 705.
- Molas M, Gomez-Valadés AG, Vidal-Alabró A, Miquel-Turu M, Bermudez J, Bartrons R, et al. (2003). Receptor mediated gene transfer vectors: Progress towards genetic pharmaceuticals. *Curr Gene Ther*, 3, 468–85.
- Peden CS, Burger C, Muzyczka N, Mandel RJ. (2004). Circulating anti wild type adeno associated virus type 2 (AAV2) antibodies inhibit recombinant AAV2 (rAAV2)-mediated, but not rAAV5 mediated, gene transfer in the brain. *J Virol*, 78, 6344–59.
- Lu PY, Xie F, Woodle MC. (2005). In vivo application of RNA interference: From functional genomics to therapeutics. *Adv Genet*, 54, 117–42.
- Liu WG, Yao K. (2002). Chitosan and its derivatives: A promising non-viral vector for gene transfection. *J Control Release*, 83, 1–11.
- Borchard G. (2001). Chitosans for gene delivery. *Adv Drug Deliv Rev*, 52, 145–50.
- Dodane V, Vilivalum VD. (1998). Pharmaceutical applications of chitosan. *Pharm Sci Technol Today*, 1, 246–53.
- Tomihata K, Ikada Y. (1997). In vitro and in vivo degradation of films of chitin and its deacetylation derivatives. *Biomaterials*, 18, 567–75.
- Onishi H, Machida Y. (1999). Biodegradation and distribution of water-soluble chitosan in mice. *Biomaterials*, 20, 175–82.
- Rao SB, Sharma CP. (1997). Use of chitosan as a biomaterial: Studies on its safety and hemostatic potential. *J Biomed Mater Res*, 34, 21–8.
- Mao H-Q, Roy K, Walsch SM, et al. (1996). DNA-chitosan nanospheres for gene delivery. *Proc Int Symp Control Rel Bioact Mater*, 23, 401–2.
- Roy K, Mao H-Q, Leong KW. (1997). DNA-chitosan nanospheres: Transfection efficiency and cellular uptake. *Proc Int Symp Control Rel Bioact Mater*, 24, 673–4.
- Lee KY, Kwon IC, Kim YH, Jo WH, Jeong, SY. (1998). Preparation of chitosan self-aggregates as a gene delivery system. *J Control Release*, 51, 213–20.
- Kiang T, Wen J, Lim HW, Leong KW. (2004). The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. *Biomaterials*, 25, 5293–301.
- Sato T, Ishii T, Okahata Y. (2001). In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. *Biomaterials*, 22, 2075–80.
- Huang M, Fong CW, Khor E, Lim LY. (2005). Transfection efficiency of chitosan vectors: Effect of polymer molecular weight and degree of deacetylation. *J Control Release*, 106, 391–406.
- Xu S, Dong M, Liu X, Howard KA, Kjems J, Besenbacher F. (2007). Direct force measurements between siRNA and chitosan molecules using force spectroscopy. *Biophys J*, 93, 952–9.

19. Chellat F, Grandjean-laquerriere A, Naour R, Fernandes J, Yahia L, Guenounou M, et al. (2005). Metalloproteinase and cytokine production by THP-1 macrophages following exposure to chitosan-DNA nanoparticles. *Biomaterials*, 26, 961-70.
20. Katas H, Alpar HO. (2006). Development and characterization of chitosan nanoparticles for siRNA delivery. *J Control Release*, 115, 216-25.
21. Murata J, Ohya Y, Ouchi T. (1996). Possibility of application of quaternary chitosan having pendant galactose residues as gene delivery tool. *Carbohydrate polymers*, 29, 69-74.
22. Erbacher P, Zhou S, Bettinger T, Steffan AM, Remy JS. (1998). Chitosan based vector/DNA complexes for gene delivery: Biophysical characteristics and transfection ability. *Pharm Res*, 15, 1332-9.
23. Lee MK, Chun SK, Choi WJ, Kim JK, Choi SH, Kim A, et al. (2005). The use of chitosan as a condensing agent to enhance emulsion mediated gene transfer. *Biomaterials*, 26, 2147-56.
24. Howard KA, Rahbek UL, Liu X, Damgaard CK, Glud SZ, Andersen MØ, et al. (2006). RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system. *Mol Ther*, 14, 476-84.
25. Illum L, Farraj NF, Davis SS. (1994). Chitosan as a novel nasal delivery system for peptide drugs. *Pharm Res*, 11, 1186-9.
26. Verhoef JC, Junginger HE, Thanou M. (2001). Chitosan and its derivatives as intestinal absorption enhancers. *Adv Drug Deliver Rev*, 50, S91-S101.
27. Liu X, Howard KA, Dong M, Anderson MO, Rahbek UL, Johnsen MG, et al. (2007). The influence of polymeric properties on chitosan/siRNA nanoparticle formulation and gene silencing. *Biomaterials*, 28, 1280-8.
28. Calvo P, Remunan-lopez C, Vila-Jato JL, Alonso MJ. (1997). Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. *J Appl Polym Sci*, 63, 125-32.
29. Shu XZ, Zhu KJ. (2000). A novel approach to prepare tripolyphosphate/chitosan complex beads for controlled drug delivery. *Int J Pharm*, 201, 51-8.
30. Rojanarata T, Opanasopit P, Techaarpornkul S, Ngawhirunpat T, Ruktanonchai U. (2008). Chitosan-Thiamine pyrophosphate as a novel carrier for siRNA delivery. *Pharm Res*, doi: 10.1007/s11095-008-9648-6.
31. MacLaughlin FC, Mumper RJ, Wang J, Tagliaferri JM, Gill I, Hinchcliffe M, et al. (1998). Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. *J Control Release*, 56, 259-72.
32. Andersen MØ, Howard KA, Paludan SR, Besenbacher F, Kjems J. (2007). Delivery of siRNA from lyophilized polymeric surfaces. *Biomaterials*, 29, 506-12.
33. Liu WG, Zhang X, Sun SJ, Sun GJ, Yao KD, Liang DC, et al. (2003). N-alkylated chitosan as a potential nonviral vector for gene delivery. *Bioconjug Chem*, 14, 782-9.
34. Wolfert MA, Seymour LW. (1996). Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block copolymers. *Human Gene Ther*, 3, 269-73.
35. Mao H-Q, Roy K, Troung-Le VL, Janes KA, Lin KY, Wang Y, August JT, Leong KW. (2001). Chitosan-DNA nanoparticles as gene carriers: Synthesis, characterization and transfection efficiency. *J Control Release*, 70, 399-421.
36. Boussif O, Lezoualch F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc Natl Acad Sci USA*, 92, 7297-301.
37. Cho YW, Kim JD, Park K. (2003). Polycation gene delivery system: Escape from endosomes to cytosol. *J Pharm Pharmacol*, 55:721-34.
38. Ishii T, Okahata Y, Sato T. (2001) Mechanism of cell transfection with plasmid/chitosan complexes. *Biochim Biophys Acta*, 1514, 51-64.
39. Fang N, Chan V, Mao H-Q, Leong KW. (2001) Interactions of phospholipid bilayer with Chitosan: effect of molecular weight and pH. *Biomacromolecules*, 2, 1161-8.
40. Dung TH, Lee S-R, Han S-D, Kim SJ, Ju YM, Kim MS, et al. (2007). Chitosan-TPP nanoparticle as a release system of antisense oligonucleotide in the oral environment. *J Nanosci Nanotechnol*, 7, 3695-9.

Copyright of Drug Development & Industrial Pharmacy is the property of Taylor & Francis Ltd and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.