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Sub-100 nm Chitosan-Triphosphate-DNA Nanoparticles for Delivery of DNA Vaccines

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Abstract: Intramuscular delivery is one of the main route for DNA vaccines administration. However, it requires large amounts of the DNA administered and external stimulation to encourage the internalization of the DNA. In this work we consider routes for less invasive administration route, and develop drug delivery systems (DDS) for intranasal administration. Chitosan polyplexes using sodium tripolyphosphate (TPP) as a crosslinker were prepared using the ionic gelation method. Our method allowed preparation of nanoparticles with the size bellow 50nm that is at least two times lower than previously reported. Moreover, despite small size, we obtained DNA encapsulation efficiencies about 70%. Parameters that may affect the encapsulation efficiency were investigated, including different TPP-chitosan ratios and concentrations of DNA. We found that encapsulation efficiency of DNA inside the particles decreases with the increasing TPP-chitosan ratio. Moreover, increasing the DNA concentration leads to a higher encapsulation efficiency. Small (<50nm) chitosan nanoparticles hold enormous potential as DNA carriers through physiological barriers and subsequent internalization.

Keywords: chitosan; DNA vaccines; tripolyphosphate; nanoparticles; ionic gelation

1. Introduction

Cancer is the second most prevalent cause of death in the world that does not have an universal cure. Human papilloma virus (HPV) is among the main carcinogenic pathogens and its infection is related with several cancers, such as cervical and oropharyngeal [1,2]. Cervical cancer is the 4th largest cause of cancer in women worldwide [3]. The prophylactic vaccination against the human papilloma virus (HPV) infection that is already commercially available, Gardasil®, from Merck, prevents the infection from HPV-16 and 18 which are responsible for around 70% of cervical cancers [4]. However, the current vaccine does not have a therapeutic effect against the already infected cells. That is, vaccine can only prevent the infection by the HPV, but cannot prevent the cancer development of cancers from pre-existing infections [5]. Therapeutic cancer vacines are still a major area of research.

One of the promising approaches towards therapeutic vaccines is DNA vaccination. DNA vaccination is applicable against a great range viral, bacterial and parasitic diseases, including HPV [6,7]. In comparison with the traditional vaccines, DNA vaccines have numerous promising advantages. They are simpler to synthesize and can be produced at a large scale. In general, they are considered to be safer, since the pathogen is not required in the vaccine production. Additionaly, DNA vaccines do not require refrigeration for storage, transport and distribution, that may allow their easier distribution worldwide [8,9]. DNA vaccines are particularly appropriate for antitumor

and anticancer treatment due to the fact that their encoded antigen can be expressed inside the antigen presenting cells (APC). These cells can then activate the needed immune response for the dissolution or destruction of a recognized infected cell [10,11]. However, the delivery of the DNA vacines to the APCs remain challending. In this work we evaluate ionotropic chitosan gelation as a potential method for the encapsulation and delivery of the DNA vaccines.

Successful drug delivery systems (DDS) should provide number of features, such as the ability to penetrate through several anatomical barriers, sustained and controlled release of their active pharmaceutical ingredients (APIs) locally, stability and deep tissue penetration. These features can be enabled by nanometer sized carriers made of synthetic and natural polymers. Nanocarriers can also be used for drugs delivery through mucosal layers, and help the cellular internalization of the API [12,13]. Ionotropic gelation is one of the most common methods used for the encapsulation and delivery of DNA. The nanoparticles are produced by mixing the ionic crosslinker, tripolyphoshate (TPP), with the cationic polymer, chitosan (CS), under constant magnetic stirring. Chitosan is known as a good carrier for delivery and internalization of non-viral vectors for gene delivery. The polycationic nature of CS, enables electrostatic complexation with the DNA, which under controlled conditions would lead to the formation of DNA-CS complexes. With the optimum concentrations and other conditions nanoparticle with sizes bellow 100 nm can be formed [14–17]. However, such small particles are rarely reported.

In this work we developed and optimized the ionotropic gelation method for encapsulation of DNA in order to produce nanoparticles with size ranging from 30 to 60 nm. We demonstrated that the method is robust and reproducible and, moreover, leads to the high DNA encapsulation efficienty.

2. Materials and Methods

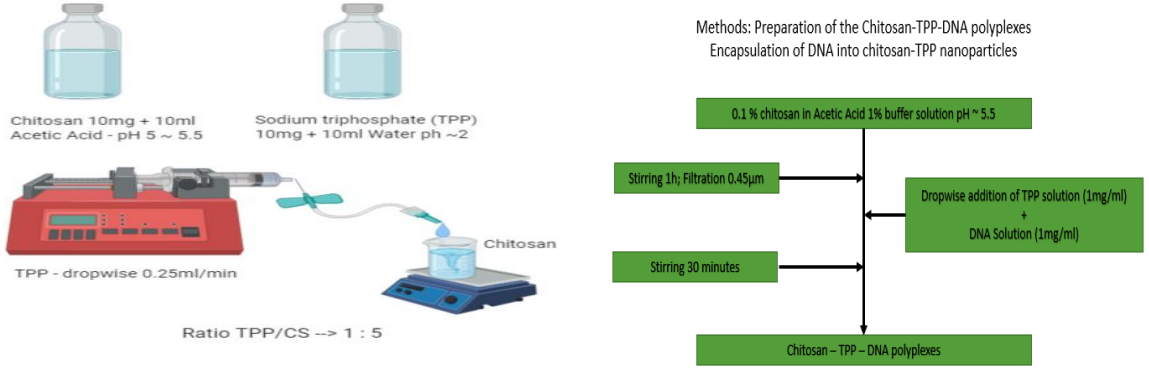
2.1. Materials

Medical grade chitosan 95/1000 was purchased from Heppe Medical, soudium tripolyphosphate (TPP) was obtained from Across Organics, deoxyribonucleic acid sodium salt (DNA) was aquired from MPBIO, 35% hydrochloric acid (HCl), sodium hydroxide palettes (NaOH) and glacial acetic acid (AA) were all acquired from VWR. The following solutions were freshly prepared by using deionized water from VWR: 2M HCl, 10M NaOH, 1% and 2% (*v*/*v*) acetic acid.

2.2. Methods2.2.1. Ionotropic Gelation

Ionotropic gelation using chitosan as a polymer and TPP as a crosslinker was used to prepare the nanoparticles. Particles with and without DNA were prepared using the same procedure adopted from [18] with slight modification. Particles without DNA were used as a negative control. The process of ionic gelation is depicted in Figure 1. 0.1% (*w*/*v*) chitosan (CS) in 1% (*v*/*v*) acetic acid and 0.1% (*w*/*v*) of TPP solution in water were prepared as stock solutions. The pH level of the chitosan and TPP solutions were adjusted to 5.2–5.5 and pH 2 by addition of NaOH and HCl respectively. For the experiments with DNA encapsulation, the DNA was dissolved in the TPP solution to reach the needed concentration. Then, both chitosan and TPP solutions were filtered using 0.45 μm polyethersulfone syringe filter (from VWR). Syringe pump (New Era Pump System, Inc., Farmingdale, NY, USA) was used to add TPP solution to the chitosan solution dropwise with the flow rate of 0.25 mL/min. To ensure consistent drop size, size 20 needle was used in all experiments, leading to the addition rate of about 15–16 drops/min. During the addition, chitosan solution was stired vigorously (600 rpm) using a magnetic stirrer. Solution was mixed for additional 30 min after all of the TPP was consumed. All experiments were done at room temperature.As a positive control, 0.1% (*w*/*v*) deoxyribonucleic acid sodium salt (DNA) in water was prepared and mixed with the blank particles without DNA in 1:1 ratio (by volume). Combined solution was incubated for 2h at room temperature for DNA adsorption to chitosan-TPP nanoparticles to take place.

All experiments were performed in triplicates and the results are presented as means ± standard deviation (SD).



(**a**) (**b**)

Figure 1. (**a**) Schematic illustration of chitosan-TPP nanoparticles (**b**) Flow chart of the nanoparticle with DNA preparation First solutions with optimized concentrations were prepared. Then TPP with DNA was added to chitosan solution dropwise while stirring.

2.2.2. Encapsulation Efficiency

To estimate the amount of the DNA encapsulated in the chitosan NPs, the NPs solution was spun down using Centurion Scientific Benchtop Centrifuge for 20 min at 22.000 RCF and the DNA concentration in the supernatant was measured. The indirect encapsulation efficience (iEE) was calculated as in (1), where c(total) is the theoretical DNA concentration in the solution and c(sup) is the measured DNA concentration after the encapsulation.

$$iE.E.\% = \frac{c(total) - \mathrm{c(sup)}}{c(total)} * 100\% \quad (1)$$

To verify the accuracy of the method, that amount of the encapsulated DNA was also estimated in the particles (dEE). NPs with and without DNA were spun down for 20 min at 22.000 RCF. The supernatant was removed for iEE measurement and the equivalent volume (1 mL) of 2% acetic acid and additional 100 µL of 2M HCl were added. The solution was vortex for one minute and then sonicated using the ultrasound homogenizer (from Qsonica sonicators) for 30 s at 40% amplitude. The solution was spun down for 20 min at 22.000 RCF mode to make sure that no pellet was formed and the DNA concentration was measured. The dEE was calculated as a ratio of measured and theoretical DNA concentrations.

In both cases DNA concentration was determine spectrophotometrically. That is, supernatant, or dissolved nanoparticle solution (1 mL) was added to the quartz cuvette. The solution was diluted 2 to 3 times with 1% acetic acid, and the absorbance was measured using a cuvette reader from SpectraMax. The absorbance value at 260 nm was recorded.

2.2.3. Particle Size Detemination

The size of the prepared CS-TPP-DNA-polyplexes was also analyzed, using the Nanophox dynamic Light Scattering (DLS) with photon cross-correlation spectroscopy from Sympatec. The particles were analyzed immediately after the preparation and after 72 h to evaluate the stability of the particle suspension. All DLS experiments were carried out at a temperature of 25 °C.

3. Results

Ionotropic gelation is a popular method for DNA encapsulation. Multiple authors have demonstrated the ability to prepared nanoparticles with DNA that are bellow 300 nm [18–20]. Liping et al. has shown that paticles smaller than 200 nm can be prepared by using solution with low chitosan

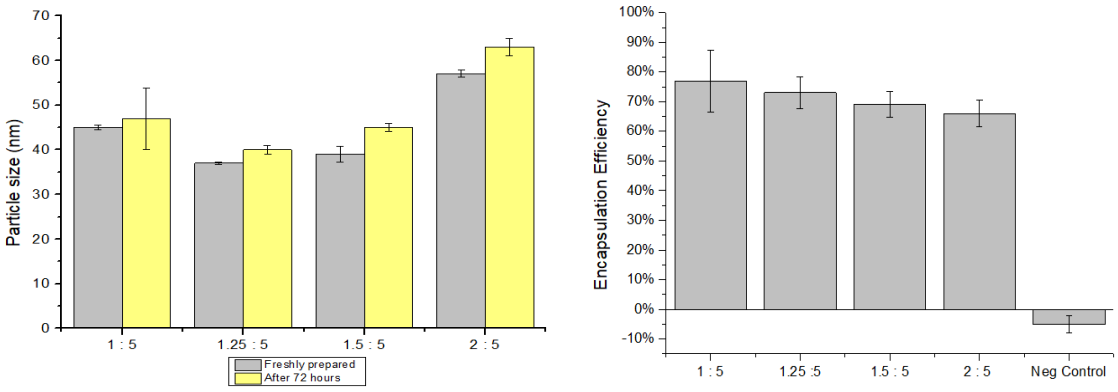
and TPP concentrations of 0.1% [19]. However, to the best of our knowledge, nanoparticles with the size bellow 100 nm have not yet been prepared. In this work we have used low solution concentrations and slow controlled drop-wise addition in order to make nanoparticles with size around 30-60 nm. We investigate the influence of the TPP/CS ratio and DNA concentration on the size and EE of formed nanoparticles.

3.1. Influence of Changing the TPP/CS Ratio on NPs Size and dEE

To investigate the influence of the TPP/CS ratio on nanoparticle size and DNA encapsulation efficiency four NPs solutions with TPP/CS ratio of 1:5, 1.25:5, 1.5:5, 2:5 were prepared. In these experiments 35 µg/mL DNA was used. The formulation of prepared samples as well as their physicochemical characteristics are summarized in Table 1. As shown in the Figure 2a, using the ionotropic gelation with controlled addition speed, very small nanoparticles of 30–50 nm were obtained. Only the particles with high TPP/CS ratio lead to formation of larger particles of about 57 nm. In all cases, the particle size did not change significantly after storing them in the frigde for 72 hrs. Only slight increase of about 2–6 nm was detected. However, as shown in Table 1 formed particles were not monodisperse and had polydispersity index > 0.5.

Table 1. Average particle size, PDI and encapsulation efficiency of chitosan-TPP-DNA polyplexes with different TPP/CS ratios.

TPP/CS Ratio	Z-Average Size (nm)	Polydispersibily Index (PDI)	Z - Average Size after 72h (nm)	(PDI) after 72h	Encapsulation Efficiency (%)
1:5	45 ± 0.5	0.55 ± 0.01	47 ± 6.9	0.51 ± 0.006	77 ± 10
1.25:5	37 ± 0.3	0.50 ± 0.03	40 ± 0.9	0.48 ± 0.004	73 ± 5
1.5:5	39 ± 1.7	0.46 ± 0.04	45 ± 0.9	0.40 ± 0.009	69 ± 4
2:5	57 ± 0.8	0.50 ± 0.02	63 ± 1.9	0.46 ± 0.010	66 ± 4



(**a**) (**b**)

Figure 2. (**a**) Particle size (nm) freshly prepared and after 72 h with different TPP/CS ratios; (**b**) Results of the encapsulation efficiency with different TPP/CS ratios.

The amount of the DNA encapsulated in the microparticles was measured using the direct method—after the extraction of the DNA from the collected nanoparticles. Prior to the measurement, the amount of the DNA that adsorbed on the surface of the NPs was evaluated by mixing the prepared blank NPs with the DNA solution and measuring the concentration of the DNA in the supernatant. 35 µg/mL of DNA was added, and −0.98 ± 1.21 µg/mL measured (not shown), indicating that only about 3% of the DNA may be adsorbed on the NP's surface. As shown in Figure 2b, negative control showed that there is no interference between the excipients used in particle preparation. As shown in Figure 2b, all formulations showed a good efficiency of encapsulation with measured dEE of 77%, 73%, 69%, 66% for samples 1 to 4 respectively. Increasing the TPP/CS ratio lead to a small decrease in the encapsulation efficiency but only of about 10%.

3.2. Influence of Changing the DNA Concentration in NP Size and dEE

To investigate the influence of the DNA concentration on the nanoparticles size and DNA encapsulation efficiency, four formulation of NPs with DNA concentration of 15, 25, 35 and 45 μg/mL were prepared. As mentioned before, particle size was measured immediately after the preparation and after 72 h to evaluate the NPs stability. The results are summarized in Table 2 and depicted in Figure 3a,b. In all cases, very small nanoparticles bellow 40 nm were formed. The particle size increased slightly after storage, yet solution can be considered stable. A small increase of about 10nm could be detected in the sample with 15 μg/mL.

Table 2. Average particle size, PDI and encapsulation efficiency of chitosan-TPP-DNA polyplexes with different DNA concentrations.

DNA Concentration (μg/mL)	Z-Average Size (nm)	Polydispersibily Index (PDI)	Z - Average Size after 72h (nm)	(PDI) after 72h	Encapsulation Efficiency (%)
15 ± 5	40 ± 0.8	0.59 ± 0.01	50 ± 4.8	0.39 ± 0.26	56 ± 28
25 ± 5	35 ± 0.5	0.48 ± 0.05	35 ± 0.5	0.53 ± 0.006	60 ± 13
35 ± 5	36 ± 0.8	0.47 ± 0.05	35 ± 0.3	0.56 ± 0.006	59 ± 4
45 ± 5	38 ± 0.7	0.47 ± 0.04	36 ± 0.4	0.55 ± 0.01	69 ± 2

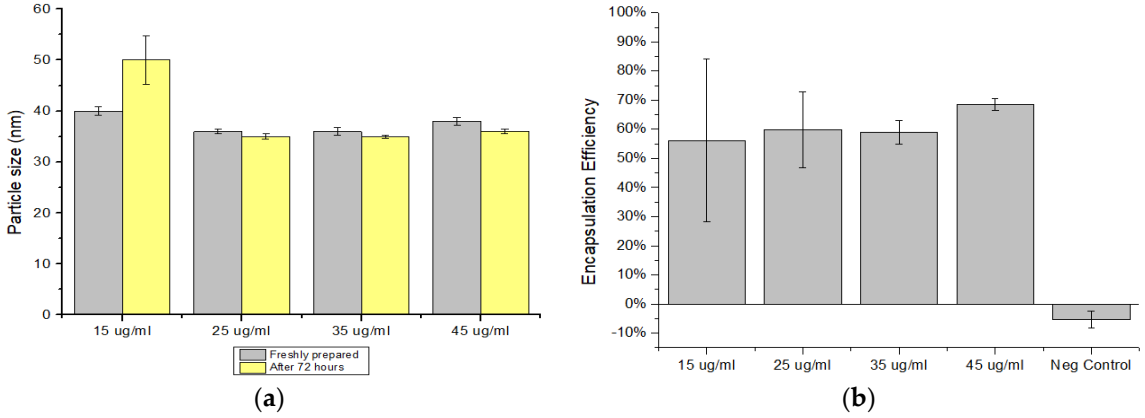


Figure 3. (**a**) Particle size (nm) freshly prepared and after 72 h with different DNA concentrations; (**b**) Results of the encapsulation efficiency with different DNA concentration.

The encapsulation efficiency measurement of nanoparticles with different amoutns of DNA added were evaluated as described before. As shown in Figure 3b, in all cases good encapsulation efficiencies were reached, dEE of 56%, 60%, 59%, 69% for samples 5 to 8, respectively. Overal, our results suggest that higher encapsulation efficiency can be obtained with the increasing concentration of DNA.

4. Discussion and Future Work

In this work, we found that increasing the TPP/CS ratio, does not influence the particle size and encapsulation efficiency this result had also been reported previously. That is, it was noted by Turan&Akbuğa [18]. Similar results were observed by Liping et al. [19]. Increasing the polymer concentration leads to an increase in particle size the influence of the polymer concentration was noted by Liping et al. [19]. The effect of chitosan molecular weight was studied by Huang et al. [20]. They have shown that low molecular weight of chitosan may lead to smaller than 70 nm nanoparticles. In this work we used low molecular weigh medical purity chitosan, exploring low solution concentrations and obtained particles that are mostly below 50 nm. The reason behind such small particle size is still under investigation. We believe that one of the factors influencing the size is the polymer mixing rate, that is controlled by both flow rate of the dropwise addition and the stirring rate. Indeed, our preliminary experiments suggest that faster addition or larger droplet size

may lead to larger nanoparticles. The influence of the addition rate on the ionotropic CS-DNA-TPP gelation has not yet been reported.

Compared to the other reported studies, we have measured slightly lower DNA encapsulation efficiencies—usually bellow 80%, Turan&Akbuğa reported around 90% of encapsulation efficiency [18]. This may be one of the drawbacks of the extremely small nanoparticles. Our further work will focus on the optimization of the CS-DNA-TPP nanoparticles for the DNA internalization in eukaryotic cells. Depending of successful results, low EE can be compensated by the easier NP uptake by targeted cells. Moreover, influence of the nanoparticle size to the mucopenetration and mucoadhesion will be tested to evaluate the feasibility of the intranasal administration.

5. Conclusions

Ionic gelation of CS/TPP nanoparticles is a promising delivery system for nucleic acids such as plasmid DNA. We have shown that these carriers can be downscalled bellow 50 nm that may enable more efficient internalization of such NPs by relevant cells. Moreover, we have shown that the good quantities of DNA can be encapsulated using the CS/TPP nanoparticles. This is especially promising for the delivery of the DNA vaccines, as high quantities of the DNA are needed to achieve the effective immunization and therapeutic effects. In further studies will be evaluated the cellular uptake and the mucopenetration/mucoadhesion of these particles.

Abbreviations

The following abbreviations are used in this manuscript:

AA: Acetic acid; API: Active pharmaceutical ingredients; APC: Antigen presenting cells; CS: Chitosan; DDS: Drug delivery systems; dEE: Direct encapsulation efficiency; DLS: Dynamic Light Scattering; DNA: Deoxyribonucleic acid; EE: Encapsulation efficiency; HCl: Hydrochoric acid; HPV: Human papilloma virus; iEE: Indirect encapsulation efficience; MHC: Major histocompatibility complex; MRT: Mean residence time; NaOH: Sodium hydroxide; NPs: Nanoparticles; pDNA: plasmid DNA; Rpm: Revolutions per minute; SD: Standard deviation; TPP: Sodium tripolyphosphate

Author Contributions: R.N. designed the study, performed the experiments, data processing, analysis of the results and wrote the paper; Â.S. helped designing the study, provided supporting material and reviewed the paper; A.S. guided the design of work and experiments, helped with the data analysis and writing of the manuscript A.A. helped with method development, supervised the work and contributed with insights and discussions; M.B. helped with the development of the experiments, discussions and ideas.

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References

1. Nagai, H.; Kim, Y.H. Cancer prevention from the perspective of global cancer burden patterns. *J. Thorac. Dis*. **2017**, *9*, 448–451. doi:10.21037/jtd.2017.02.75.
2. Doorbar, J.; Egawa, N.; Griffin, H.; Kranjec, C.; Murakami, I. Human papillomavirus molecular biology and disease association. *Rev. Med. Virol*. **2015**, *25*, 2–23.
3. Arbyn, M.; Weiderpass, E.; Bruni, L.; de Sanjosé, S.; Saraiya, M.; Ferlay, J.; Bray, F. Estimates of incidence and mortality of cervical cancer in 2018: A worldwide analysis. *Lancet Glob Health* **2020**, *8*, e191–e203. doi:10.1016/S2214-109X(19)30482-6. PMID: 31812369; PMCID: PMC7025157.
4. Padmanabhan, S.; Amin, T.; Sampat, B.; Cook-Deegan, R.; Chandrasekharan, S. Intellectual property, technology transfer and manufacture of low-cost HPV vaccines in India [published correction appears in Nat Biotechnol. 2012 Feb;30(2):193]. *Nat Biotechnol*. **2010**, *28*, 671–678. doi:10.1038/nbt0710-671.

5. Almeida, A.M.; Queiroz, J.A.; Sousa, F.; Sousa, A. Cervical cancer and HPV-Infection: ongoing therapeutic research to counteract the action of E6 and E7 oncoproteins. *Drug Discov. Today* **2019**, *24*, 2044–2057 doi:10.1016/j.drudis.2019.07.011.
6. Ha, S.; Jeon, B.; Youn, J.; Kim, S.; Cho, S.; Sung, Y. Protective effect of DNA vaccine during chemotherapy on reactivation and reinfection of Mycobacterium tuberculosis. *Gene Ther*. **2005**, *12*, 634–638.
7. Okuda, K.; Xin, K.Q.; Haruki, A.; Kawamoto, S.; Kojima, Y.; Hirahara, F.; Okada, H.; Klinman, D.; Hamajima, K. Transplacental genetic immunization after intravenous delivery of plasmid DNA to pregnant mice. *J. Immunol*. **2001**, *167*, 5478–5484.
8. Smith, H.A.; Klinman, D.M. The regulation of DNA vaccines. *Curr. Opin. Biotechnol*. **2001**, *12*, 299–303.
9. Kutzler, M.A.; Weiner, D.B. DNAvaccines: ready for prime time? *Nat. Ver. Genet.* **2008**, *9*, 776–788.
10. Anderson, R.J.; Schneider, J. Plasmid DNA and viral vector-based vaccines for the treatment of cancer. *Vaccine* **2007**, *25* (Suppl. 2), B24–B34.
11. Lee, J.; Arun Kumar, S.; Jhan, Y.Y.; Bishop, C.J. Engineering DNA vaccines against infectious diseases. *Acta Biomater.* **2018**, *80*, 31.
12. Mukherjee, S.; Ray, S.; Thakur, R.S. Solid lipid nanoparticles: A modern formulation approach in drug delivery system. *Indian J. Pharm. Sci.* **2009**, *71*, 349–358. doi:10.4103/0250-474x.57282.
13. Panyam, J.; Labhasetwar, V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv. Drug Deliv. Rev.* **2003**, *55*, 329–347.
14. López-León, T.; Carvalho, E.L.; Seijo, B.; Ortega-Vinuesa, J.L.; Bastos-González, D. Physicochemical characterization of chitosan nanoparticles: electrokinetic and stability behavior. *J. Colloid Interface Sci*. **2005**, *283*, 344–351. doi: 10.1016/j.jcis.2004.08.186. PMID: 15721903.
15. Morimoto, M.; Saimoto, H.; Usui, H.; Okamoto, Y.; Minami, S.; Shigemasa, Y. Biological activities of carbohydrate-branched chitosan derivatives. *Biomacromolecules* **2001**, *2*, 1133–1136.
16. Li, X.B.; Tsushima, Y.; Morimoto, M.; Saimoto, H.; Okamoto, Y.; Minami, S.; Shigemasa, Y. Biological activity of chitosan-sugar hybrids: specific interaction with lectin. *Polym. Adv. Technol*. **2000**, *11*, 176–179.
17. Di Martino, A.; Sittinger, M.; Risbud, M.V. Chitosan: A versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials* **2005**, *26*, 5983–5990.
18. Özbaş-Turan, S.; Akbuğa, J. Plasmid DNA-loaded chitosan/TPP nanoparticles for topical gene delivery. *Drug Deliv*. **2011**, *18*, 215–222. doi: 10.3109/10717544.2010.544688. PMID: 21226549.
19. Wang, L.; Zhang, W.; Zhou, Q.; et al. Establishing Gene Delivery Systems Based on Small-Sized Chitosan Nanoparticles. *J. Ocean Univ. China* **2018**, *17*, 1253–1260. doi:10.1007/s11802-018-3658-8.
20. Huang, K; Sheu, Y; Chao, I. Preparation and Properties of Nanochitosan. *Polym. -Plast. Technol. Eng.* **2009**, *48*, 1239–1243, doi:10.1080/03602550903159069.