# HYDROGEL NANOPARTICLE ENCAPSULATED PLASMID AS A SUITABLE GENE DELIVERY SYSTEM

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*To facilitate the delivery of genetic material, the use of appropriate carriers such as polymers is necessary. Nanoparticles comprising of chitosan-alginate polymers were formed through pregel preparation method. Chi/Alg nanoparticles had a mean Z-Average diameter of 161.8 nm and mean zeta 29.3 mV, respectively. The ability of plasmid-complex in preventing DNA migration showed Chi/Alg nanoparticles have great capacity to maintain plasmid. The efficiency of nanoparticles for transfection of pEGFP-N1 plasmid in the cultured HEK 293 cells was measured by flow cytometry. Cell viability assays indicated that nanoparticles had no toxic effect on HEK 293 cells after 4 or 24 h. Our suitable candidate for gene delivery would be Alg/Chi nanoparticles.*

***Key words:** chitosan, alginate, nanoparticle, size, transfection efficiency, cytotoxicity.*

**Introduction.** The basic concept underlying gene therapy is the use of DNA as a pharmaceutical agent for curing diseases. The most currently used gene delivery systems are pharmaceutical nanocarriers, such as polymeric nanoparticles [1–3]. These biodegradable polymers demonstrate useful properties such as non-toxicity, stability and protect gene from degradation and etc [4].

Chitosan and alginate are the most commonly studied polymers in recent years.

Chitosan (Chi) is a cationic polymer which can complex with DNA via electrostatic interactions between anionic phosphate of DNA and amine groups of chitosan [5] and is successfully used as a non-viral gene delivery system both in vitro and in vivo [6, 7]

Alginate (Alg) is a kind of hydrophilic polysaccharide [8]. Polyanion/polycation cross-linked hydrogel based on alginate and chitosan have gained much attention in recent years as carriers for local drug delivery [9].

Chitosan-alginate (Chi/Alg) polyionic complexes are formed through the ionic gelation via interactions between the carboxyl groups of alginate and the amine groups of chitosan [10–13]. The main purpose of this study was to determine the size and physicochemical properties of Chi/Alg – pEGFP-N1 complexes. Then the potential of these nanoparticles in cell transfection, preventing DNA migration and their stability in aqueous medium were analyzed. The ability of plasmid-complexes on cell viability was also determined.

**Materials and methods.** *Materials.* Sodium alginate (Sigma), Low molecular weight chitosan (Sigma-Aldrich Co., Germany), L-gluthamine, Penicillin, Streptomycin, Fetal bovine serum (FBS) (Biosera, South Korea), PolyFect transfection reagent (Qiagen, Germany), $^{3}$H-thymidine (Amersham, UK).

*Chitosan-alginate nanoparticles preparation for particle size and zeta potential analysis.* Chi/Alg nanoparticle preparation of N/P ratio of 5, $CaCl_2$/Alg ratio of 0.2 %, Chi/Alg ratio of 1 and pH 5.3 was performed according to Gazori et al. [14].

Separate stock solutions of 1 mg/ml sodium alginate and calcium chloride were prepared and 25 mg of chitosan was dissolved in 25 ml of deionized water of 1 % acetic acid solution.

Subsequently, plasmid was allowed to complex with chitosan by vortexing 10 ng plasmid. pEGFP-N1 was prepared with 200 ng/μl concentration with 130 μl (130 μg) of chitosan from the stock solution mentioned above. The final solution was diluted up to 1 ml with deionized water and then incubated for 30 min in room temperature. 130 μl of sodium alginate from the stock solution was diluted with up to 3 ml filtered deionized water. Then in the case of $CaCl_2$/Alg ratio of 0.2 %, 26 μl calcium chloride

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solution was diluted with filtered deionized water up to 1 ml and then added drop wise to alginate solution with the final volume of 4 ml under magnetic stirring condition for 10 min.

Chitosan-plasmid solution was then added drop wise to aforementioned solution for further 30 min under stirring condition at room temperature. The particle suspension was then centrifuged in Amicon® Ultra-10 (Ultracel-Sok) tube at 4000 rpm for 30 min.

Scanning electron microscopy (LEO1455 VP, 10KV Cambrige) was used for particle size and morphological characteristic analysis. The particle size and zeta potential were also detected by using Scattering Particle Analyzer and Malvern zeta sizer ZS series, respectively (Malvern, Co., UK).

*Gel electrophoresis of nanoparticles.* According to the above-mentioned method with the Chi/Alg nanoparticles encapsulating 3 μg of plasmid were prepared. After centrifugation at 25 °C in Amicon® Ultra-10 centrifuge tube, the nanoparticles were all incubated in aqueous medium for 1, 3, 5, 7, 9 and 10 h. 30 μl of samples were mixed with loading dye and were electrophoresed for 70 min run at 120 V on a 2 % agarose gel and the gel was photographed using gel documentation (Vilber lourmant, Germany).

The ability of chitosan/alginate complex to protect the plasmid against chitosanase and lysozyme were also evaluated. Naked plasmid, chitosan/alginate nanoparticle-DNA complex were treated with 1UDNase I for 15 min at 37 °C, followed by heat inactivation (60 °C for 15 min) in the presence of 25 mM EDTA. Nanoparticles were then digested through incubation (4 h, 37 °C) with 10 μL chitosanase (48 U/mL in 50 mM acetate buffer pH 5.5) and 8 μL lysozyme (0.5 U/mL in 50 mM acetate buffer pH 5.5) [1]. Samples were then run on a 1 % agarose gel.

*Determination of transfection efficiency by flow cytometry.* One day before experiment, HEK 293 cells (Pasteur Institute Cell bank of Iran) were cultured in 6-well plates at $6 \cdot 10^5$ cells/well in RPMI supplemented with 10 % fetal bovine serum (FBS), 2.0 mM L-gluthamine, 100 U/ml penicillin and 100 μg/ml streptomycin in 37 °C, 5 % humidified $CO_2$ incubator.

On the day of transfection, the medium was removed and the Chi/Alg nanoparticles (26 μg/μl Chi/Alg and 2 μg plasmid were used in all cases)

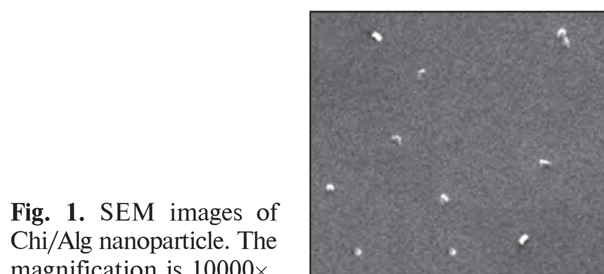

**Fig. 1.** SEM images of Chi/Alg nanoparticle. The magnification is 10000×

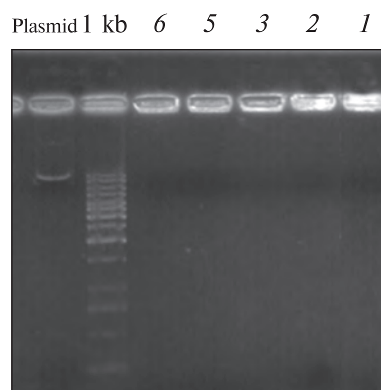


**Fig. 2.** Agarose gel electrophoresis of Chi/Alg – pEGFP-N1 complexes. Samples were run on a 2 % gel: *1* – pEGFP-N1 plasmid; *2* – 1-kb ladder; *3, 4, 5, 6, 7* – Chi/Alg – pEGFP-N1 complex after 6, 5, 3, 2 and 1 h incubation

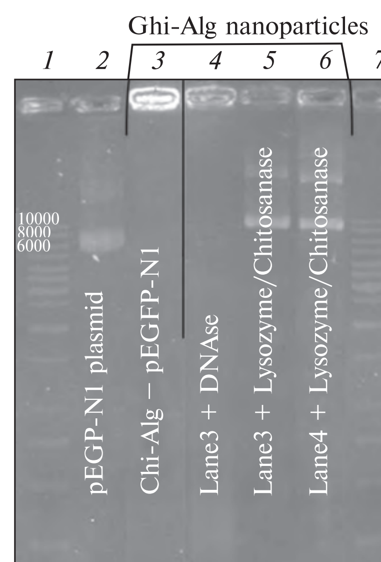


**Fig. 3.** Agarose gel electrophoresis of Alginate-chitosan – plasmid complexes. Samples were run on a 1 % gel: *1, 7* – 1-kb ladder; *2* – pEGFP-N1 plasmid; *3* – Chi-Alg – pEGFP-N1; *4–6* – Chi/Alg – pEGFP-N1 following DNAse I, chitosanase/lysozyme and chitosanase/lysozyme/DNAse I digestion

**Fig. 4.** Fluorescent microscopy imaging of the transfected cells as 400× magnification: *a* – the transfected HEK 293T cells showed the GFP expression for polyfect; *b* – GFP expression for Chi/Alg nanoparticles; *c* – the cells in visible light

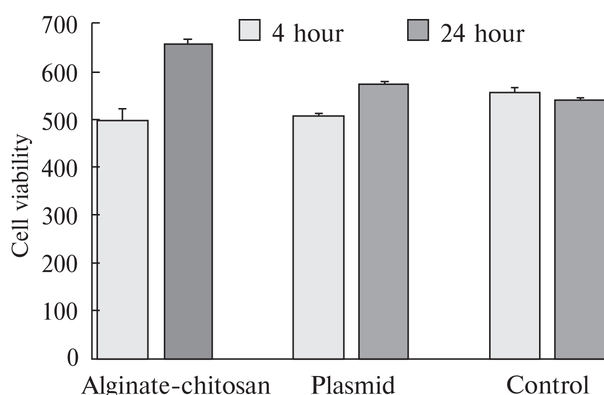


**Fig. 5.** Cytotoxicity of alginate-chitosan-plasmid nanoparticles compared with plasmid alone and control. The HEK 293 cells were cultured in 96-well plates and were exposed to 10 μg plasmid and Chi/Alg – pEGFP-N1 nanoparticles (26 μg/ml) and negative control (medium cell culture (RPMI)). All data are presented as mean of three different measurements ± SE

were added to the cells. Polyfect ™ (10 μl) loaded with 10 ng pEGFP-N1 was also used as a control. At 48 h post-transfection, the medium was removed and the cells were washed with buffered saline (PBS). Expression of EGFP was first visualized by fluorescent microscopy (Leitz Germany) and the transfection efficiency percentage was determined by flow cytometry (BD, FACScan).

*Cytotoxicity assay and statistical analysis.* About $1 \cdot 10^4$ cells/well HEK 293 cells were cultured in 48-well plate in 180 μl of complete growth medium. After 48 h, Chi/Alg – pEGFP-N1 nanoparticles (26 μg/ml) and 10 μg pEGFP-N1 alone was added to the cells in triplicate with for 4 and 24 h. Control cells were treated only with the culture medium. After exposure times, all the media were replaced with fresh complete growth medium and the plates were incubated at 37 °C and 5 % $CO_2$. One μCi/ml $^3$H-thymidine (Sigma, USA) was added to the cells for a further 16 h. Finally, the incorporated radioactive label activity was determined using a beta-counter (Wallac). Statistical significance was determined using students' two-sided *t*-test with $p < 0.05$ deemed significant.

**Results and discussion.** *Zeta potential analysis of the nanoparticles.* Nanoparticles of Chi/Alg had an average size of 161.8 nm (Fig. 1) and a strongly positive charge of +29.3 mV.

According to Rieux 2006, the optimum size of nanoparticles that can be absorbed by enterocytes is 100–200 nm [15] which shows that our Chi/Alg nanoparticles (161 nm) are also in appropriate size for mucosal absorption. Indeed, the majorities of amine groups of chitosan were protonated at pH 5.3 and were able to participate in electronic interactions with carboxyl group of alginate. Here, alginate can be cross-linked with the pEGFP-N1 using polyvalent cations such as $Ca^{2+}$ [16, 17].

*Gel electrophoresis of nanoparticles.* Electrophoresis analysis was conducted to assess the stability of Chi/Alg – pEGFP-N1 (*1*, *2*, *3*, *5* and *6*) after synthesis. It can be seen from Fig. 2 that pDNA wrapped in Chi/Alg nanoparticles was unable to move and remained in the loading wells even after 6 h post synthesis.

For the purpose of evaluating the ability of Chi/Alg nanoparticles to protect DNA, its integrity was evaluated following complexation and release from nanoparticleas, with and without exposure to nucleases. Chi/Alg nanoparticles showed capacity of protecting DNA from enzymatic digestion (Lane *4*, *5*, *6*). As illustrated in Fig. 3, plasmids complexed with Chi/Alg nanoparticles were protected from digestion, as demonstrated by the release of plasmid following inactivation of Dnase I and degradation of the nanoparticles (Lane *5*, *6*).

*In vitro transfection.* Cellular uptake of Chi/Alg nanoparticles was investigated using the green fluo-

rescent protein plasmid (pEGFP-N1 – 4.7 kb) and were compared to polyfect transfection. Two days after transfection, the cells emitted the green fluorescence (Fig. 4, *a*) under the immunofluorescent microscope.

Flow cytometric analysis of the transfected cells indicated 68 and 42 % GFP expression for polyfect and Chi/Alg nanoparticles, respectively. The results show that nanoparticles transfection and expression efficiency was less than polyfect/plasmid.

In fact, positive charge of amine groups of chitosan can complex with negative charge of DNA phosphate which results in low gene transfection efficiency [18]. Therefore incorporation of negatively charged polymer such as alginate decreases the strength of interaction between DNA and chitosan. This should increase the transfection efficiency of the system by facilitating the release of DNA once the particles have been internalized by cells [19, 20]. However, other mechanisms of endosomal release such as swelling of the polymer due to its hydrogel effect may increase the release of pEGFP into the cytosol [21–23].

*Toxicity of nanoparticles.* The effect of the nanoparticle-plasmid complexes on the cell viability was determined using cytotoxicity assay. As illustrated in Fig. 5, Chi/Alg nanoparticles showed no toxicity and no difference compared to the control after 4 h but had higher cell viability after 24 h ($p < 0.05$). These results showed that alginate and chitosan increased the cell proliferation by influencing on mitochondrial activity of the cells, stimulating them to proliferate [21].

**Conclusion.** In this study Chi/Alg nanoparticles have prepared with Chi/Alg ratio of 1, $CaCl_2$/Alg ratio of 0.2 % and N/P ratio of 5 at pH 5.3. The great amine groups of chitosan were protonated at pH 5.3 and had electrostatic interactions with plasmid which could limit the release of pDNA by chitosan. Therefore Chi/Alg nanoparticles mediated transfection of HEK cells, resulted in 42 % transfection. This shows the effect of alginate on reducing the strength of electrostatic interactions between chitosan and pDNA, resulting in better transfection and increasing the plasmid release.

Moreover, an average size of Chi/Alg nanoparticles was 161.8 nm with mean zeta potential of 29.3 mV, providing characteristics desirable for gene delivery. Also unlike the intact pDNA, pDNA encapsulated in Chi/Alg nanoparticles weren't released after 6 h which makes it a good candidate for pDNA protection.

## ПЛАЗМИДЫ, ИНКАПСУЛИРОВАННЫЕ В ГИДРОГЕЛЬНЫЕ НАНОЧАСТИЦЫ, КАК ПОДХОДЯЩАЯ СИСТЕМА ДОСТАВКИ ГЕНОВ

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Для облегчения доставки генетического материала необходимо использование подходящих носителей, таких как полимеры. Наночастицы, состоящие из хитозан-альгинатных полимеров, были получены методом подготовки прегеля. Chi/Alg наночастицы имели средний диаметр 161.8 нм (Z-Average) и средний zeta-потенциал 29.3 mV. Отсутствие миграции ДНК во время электрофореза комплексов плазмиды с наночастицами показало, что Chi/Alg наночастицы могут удерживать плазмидную ДНК внутри комплекса. Эффективность наночастиц для трансфекции плазмиды pEGFP-N1 в культивируемые клетки HEK 293 была измерена с помощью жидкостной цитометрии. Тесты на жизнеспособность клеток показали, что наночастицы не имели токсичного эффекта на клетки HEK 293 через 4 ч или 24 ч. Наночастицы Alg/Chi являются подходящим кандидатом для доставки генов.

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