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Comparison of chitosan, alginate and chitosan/alginate nanoparticles with respect to their size, stability, toxicity and transfection

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PEER REVIEW

Peer reviewer

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Comments

This study is interesting as the authors have compared the most important factors of nanoparticles on the most popular kinds of nanoparticles. Findings may help towards the production of more efficient gene delivery vehicles.

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ABSTRACT

Objective: To to compare the chitosan/alginate, chitosan and alginate nanoparticles as plasmid vectors, to determine the morphological characteristics, size and physicochemical properties of nanoparticle–pEGFP complexes and to evaluate the potential of these nanoparticles in transfection of pEGFP plasmid in to a cultured the human embryonic kidney cell line (HEK 293 cells).

Methods: Nanoparticles comprising chitosan, alginate and both chitosan–alginate polymers were formed through pregel preparation method. The ability of plasmid–complexes in preventing DNA migration were assessed by the agarose gel assay. The efficiency of nanoparticles in transfection of pEGFP plasmid in the cultured HEK 293 cells was measured by flow cytometry. The effect of the nanoparticle–plasmid complexes on the cell viability was determined using cytotoxicity assay.

Results: Chitosan, alginate and alginate/chitosan nanoparticles had a mean Z–average diameter of 620 nm, 235.8 nm and 161.8 nm and mean zeta potential of 45 mV, −18.6 mV and 29.3 mV, respectively. Chitosan and chitosan/alginate nanoparticles have greater capacity to maintain plasmid than alginate nanoparticles. Alginate nanoparticles had the greater transfection in comparison to the others. Cell viability assays indicated that nanoparticles had no toxic effect on HEK 293 cells after 4 h or 24 h.

Conclusions: The combination of particle surface, hydrophobicity size and zeta potential can influence on transfection efficiency and the cellular uptake of the nanoparticles. Our suitable candidate for gene delivery would be alginate/chitosan nanoparticles.

KEYWORDS

Alginate, Cell culture, Chitosan, Cytotoxicity, Gene therapy, Morphological characterization, Nanoparticle, Plasmid DNA , Transfection efficiency, Zeta potential

1. Introduction

In recent years, significant researches have been devoted to gene therapy. Plasmid DNA is utilized in gene therapy or as a component of a vaccine. In clinical applications, plasmid DNA has the ability to treat many diseases via modifying a gene expression[1–3]. However, barriers including low transfection or degradation of naked genes by nucleases and restriction enzymes are the main reasons to formulate plasmid DNA into nanoparticles[4].

Non–viral systems such as polymeric systems have the potential to deliver drugs or biomolecules due to their

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properties such as safety, non toxicity, protection of the drug, biodegradability, delivering gene to target tissue and the ease of production in large quantities[5].

Compared with other polymers, chitosan and alginate have been particular appealing as a gene vector in recent years[6].

Chitosan is a biodegradable cationic polysaccharide which is composed of D-glucosamine and N-acetyl-D-glucosamine. Its cationic polyelectrolyte nature provides a strong electrostatic interaction with negatively charged DNA[7,8].

Chitosan and its derivatives interact with DNA by ionic interactions between anionic phosphate backbones of DNA and primary amine groups of chitosan. This binding protects DNA from degradation and increases the transfection efficiency compared to the naked DNA[9].

Coating chitosan by anionic polymers such as alginate can reduce the strength of the interaction between chitosan and DNA. This status will result in less stable particle, improve the transfection efficiency and improve its dissociation within the cell[10-13].

Alginate is a co-polymer, consisting of linear chains of α-L-glucuronic acid (G) and β-D-mannuronic acid (M) produced by marine brown algae. It is a useful biopolymer to prepare nanocapsules due to its good biocompatibility, biodegradability, non-toxicity and mucoadhesion properties[14-16].

Guluronic acids of alginate have the ability to exchange their Na^+ ion and react with Ca^{2+}. In this reaction, the α-L-guluronic acid groups will connect to each other by these divalent cations. Dimerization of alginate chains will also help them to join with many other chains which results in a gel network[17].

The main purpose of this study was to compare the chitosan/alginate, chitosan and alginate nanoparticles as plasmid vectors. The morphological characteristics, size and physicochemical properties of nanoparticle-plasmid enhanced green florescence protein (pEGFP) complexes were first determined. Then the potential of these nanoparticles in transfection of pEGFP plasmid into a cultured human embryonic kidney cell line (HEK 293 cell) was evaluated.

The ability of plasmid-complexes in preventing DNA migration and the effect of nanoparticle-plasmid complexes on cell viability were also determined.

2. Materials and methods

2.1. Materials

Polymers for nanoparticle preparation included low molecular weight chitosan (Sigma-Aldrich Co., Germany) and sodium alginate (Sigma), calcium chloride (Sigma), L-gluthamine, penicillin, streptomycin, fetal bovine serum (Biosera, South Korea), PolyFect transfection reagent (Qiagen, Germany), ^3H-thymidine (Amersham, UK).

2.2. Preparation of nanoparticles

2.2.1. Stock solutions

Stock solutions of sodium alginate and calcium chloride were 0.1% w/v in deionized water. About 25 mg of chitosan was then dissolved in 25 mL of deionized water of 1% acetic acid solution (250 μL acetic acid). pEGFP-N1 plasmid was dissolved with diethylpyrocarbonate water to prepare a 200 ng/μL concentration stock solution. All the stock solutions were filtered (0.22 μm syringe filter) prior to use.

2.2.2. Preparation of alginate-chitosan, chitosan and alginate nanoparticles

In preparing alginate-chitosan-plasmid nanoparticles, the optimum condition for preparation of alginate-chitosan nanoparticles was alginate-chitosan ratio of 1, $CaCl_2$/alginate ratio of 0.2% and N/P ratio of 5 at pH 5.3[18]. Nanoparticles were prepared under sterile condition. With respect to N/P and alginate-chitosan ratio, 130 μL (130 μg) of sodium alginate stock solution was diluted with up to 3 mL filtered deionized water. Then 26 μL calcium chloride solution adjusted to alginate/$CaCl_2$ percentage ratio was diluted with filtered deionized water up to 1 mL and then added dropwise to alginate solution with the final volume of 4 mL under magnetic stirring condition for 10 min.

Chitosan-plasmid solution was prepared by vortexing 10 ng plasmid (pEGFP-N1 plasmid was prepared with 200 ng/μL concentration) with chitosan (equal amount sodium alginate) up to 1 mL with deionized water. This solution was then added dropwise to aforementioned solution for further 30 min under stirring condition.

The particle suspension was then centrifuged at 20° C in Amicon® Ultra-10 (Ultracel- Sok) centrifuge tube at 4000 r/min for 30 min to separate free polymers from the nanoparticles.

In preparing chitosan-plasmid nanoparticles, chitosan coated nanoparticles were obtained by adding 130 μL chitosan stock solution to 4 mL deionized water. Then 10 ng plasmid, diluted with up to 1 mL filtered deionized water, were added dropwise to the above solution under stirring condition. The particle suspension was then centrifuged at 20° C in Amicon® Ultra-10 (Ultracel- Sok) centrifuge tube at 4000 r/min for 30 min.

Alginate-plasmid nanoparticles were prepared by adding 130 μL of sodium alginate stock solution (0.1% w/v) with up to 3 mL deionized water. Then 26 μL $CaCl_2$ solution adjusted to $CaCl_2$/alginate ratio was added dropwise to alginate solution under stirring condition for 10 min, followed by addition of 10 ng plasmid dropwise to the alginate/$CaCl_2$ solution (both was diluted with filtered deionized water up to 1 mL). The particle suspension was then centrifuged at 20° C in Amicon® Ultra-10 (Ultracel- Sok) centrifuge tube at 4000 r/min for 30 min.

2.3. Morphological characterization, size and surface charge study

Morphological characteristics of nanoparticle were examined by scanning electron microscopy (LEO1455 VP, 10KV Cambrige). The particle size and zeta potential were detected by using scattering particle analyzer and Malvern Zetasizer ZS series, respectively (Malvern, Co., UK).

2.4. Gel electrophoresis of nanoparticles

For the purpose of investigating the stability of plasmid loaded nanoparticles in aqueous medium, nanoparticles of alginate-

chitosan, alginate and chitosan were prepared according to the above–mentioned methods. A total of 3 μg of plasmid was used in all cases. Each suspension was centrifuged in Amicon® Ultra–10 centrifuge tube to separate free polymers from the nanoparticles. The three series of loaded nanoparticles were all incubated in aqueous medium for 5 h. In each case, 30 μL of samples were mixed with loading dye and were run on a 2% agarose gel at 120 V for 60 min and the gel was photographed using gel documentation (Vilberlourmant, Germany).

The ability of alginate–chitosan, chitosan and alginate complexes in protecting the plasmid against chitosanase and lysozyme were also examined. Naked plasmid, alginate–chi tosan nanoparticle–plasmid complex, alginate nanparticle–plasmid complex and chitosan nanoparticle–plasmid complex were treated with 1 IU DNase I for 15 min at 37 °C, followed by heat inactivation (60 °C for 15 min) in the presence of 25 mmol/L ethylene diamine tetraacetic acid. Nanoparticles were then digested through incubation (4 h, 37 °C) with 10 μL chitosanase (48 IU/mL in 50 mmol/L acetate buffer pH 5.5) and 8 μL lysozyme (0.5 IU/mL in 50 mmol/L acetate buffer pH 5.5). Samples were then run on a 1% agarose gel.

2.5. Cell culture and in vitro transfection

HEK 293 cell was obtained from Pasteur Institute Cell bank of Iran (Tehran, Iran). These cells were cultured in 6–well plates at 6×10^5 cells/well in Roswell Park Memorial Institute medium (RPMI) supplemented with 2.0 mmol/L L–gluthamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum, incubated at 37 °C, 5% CO_2.

The following day, the medium was removed and the cells in each of the three wells were treated with chitosan and alginate nanoparticles (26 μg/μL alginate–chitosan and 2 μg plasmid were used in all cases). Polyfect™ (10 μL) loaded with 10 ng plasmid was also used as a control. At 48 h post–transfection, the cells were washed with phosphate buffer solution. Expression of EGFP was first visualized by fluorescent microscopy (Leitz Germany) and then the percentage of the transfection efficiency was determined by flow cytometry (BD, FACScan).

2.6. Cytotoxicity assay

HEK 293 cells were seeded in 48–well plate at 1×10^4 cells/well in 180 μL of complete growth medium. After 48 h, the cells in triplicate were treated with 10 μg plasmid alone, alginate–chitosan nanoparticles (26 μg/mL), alginate and chitosan nanoparticles (26 μg/mL) alone 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. About 1 μCi/ml ^{3}H–thymidine (Sigma, USA) was added to the cells for the final 16 h of the culture. Cells were harvested onto glass fiberfilters (Wallac, Lund, Sweden) and the incorporated radioactive label activity was determined using a beta–counter (Wallac).

2.7. Statistical analysis

Each treatment was repeated three times in triplicate format and results were expressed as means±SE. Statistical significance was determined using students two–sided *t*–test with $P<0.05$ deemed significant.

3. Results

3.1. Nanoparticle size and zeta potential

In alginate–chitosan nanoparticles preparation, the pH was set at 5.3. Chitosan and alginate nanoparticles had an average size of 620 nm and 235.8 nm and the mean zeta potential of 45 mV, −18.6 mV, respectively.

The smallest nanoparticles were made of alginate–chitosan with an average size of 161 nm (Figure 1) and the mean zeta potential of 45 mV similar to Gazori's finding[18].

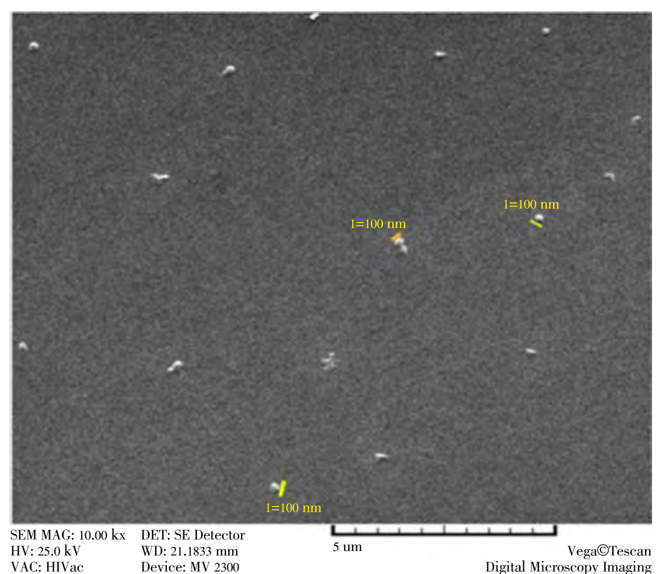

Figure 1. SEM images of alginate/chitosan nanoparticle produced with alginate/chitosan ratio of 1, $CaCl_2$/alginate ratio of 0.2% and N/P ratio of 5 at pH 5.3. The magnification is 10 000×.

3.2. Gel electrophoresis of nanoparticles

The ability of the plasmid–complexes in preventing DNA migration was assessed by the agarose gel assay. While plasmid released from alginate nanoparticles, chitosan–plasmid and chitosan–alginate–plasmid showed no plasmid release after 5 h (Figure 2). Alginate–chitosan nanoparticles showed capacity of protecting DNA from enzymatic digestion (Figure 3, Lane 6, 7, 8). As illustrated in Figure 3, plasmids complexed with chitosan/alginate nanoparticles were protected from DNase I digestion. Conversely chitosan/pEGFP nanoparticles and alginate/pEGFP nanoparticles couldn't protect the plasmid againt DNase I degradation.

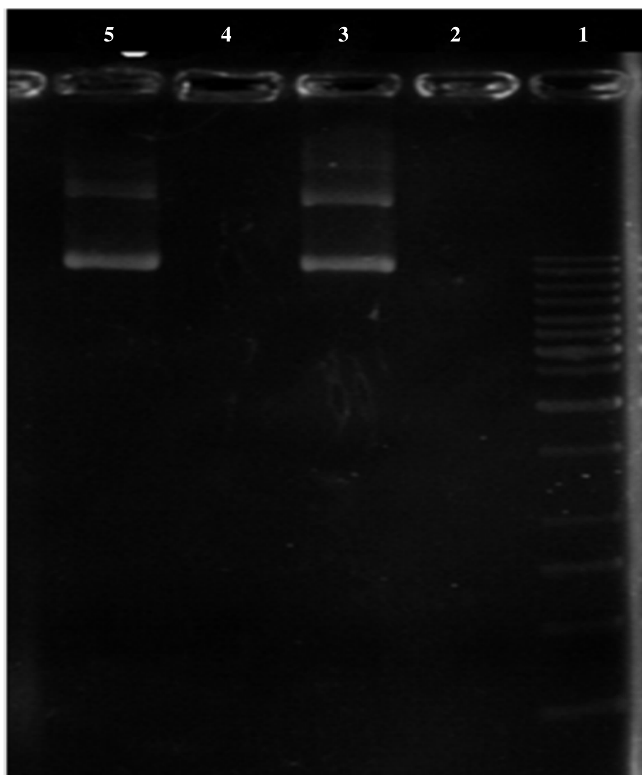

Figure 2. Agarose gel electrophoresis of plasmid, chitosan and alginate-chitosan nanoparticles to determine degree of complexation.

Samples were run on a 2% gel. Ladder 1 Kb (lane 1), alginate/chitosan (lane 2), pEGFP (lane 3), chitosan alone (lane 4) and alginate nanoparticles (lane 5). Chitosan and chitosan/alginate nanoparticles demonstrated greater capacity to maintan plasmid than alginate nanoparticles.

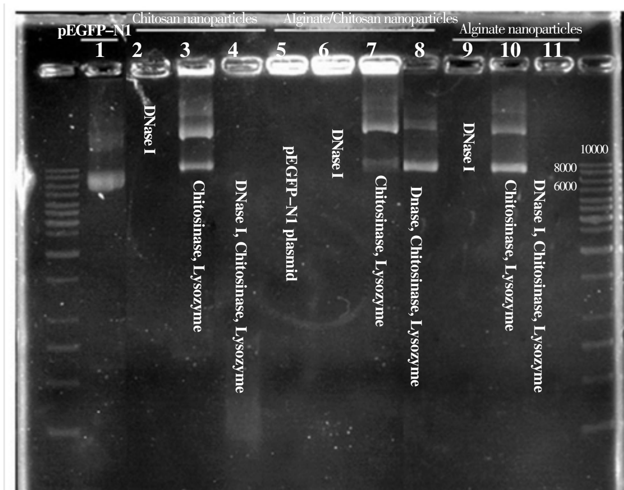

Figure 3. Agarose gel electrophoresis of alginate/chitosan, chitosan and alginate nanoparticles following DNase digestion and treatment by chitosinase/lysozyme to digest particles.

Samples were run on a 1% gel. About 1 kb ladder were loaded at the right and left ended of gel: pEGFP-N1 plasmid (Lane 1). Chitosan and alginate nanoparticles alone couldn't preserve activity of pEGFP-N1 againt DNase degradation (Lane 2, 9). Complexation with alginate/chitosan/pEGFP-N1 nanoparticles preserves activity of DNA (Lane 6).

As showed in lane 7 (Figure 3), plasmids complexed with chitosan-alginate nanoparticles were released after inactivation of DNase I and degradation of nanoparticles by chitosinase and lysozyme but plasmids. The results were conflict for alginate/pEGFP and chitosan/pEGFP nanoparticles (Figure 3, lane 4, 11).

All three kinds of nanoparticles ptotected plasmid against chitosinase and lysozyme digestion.

3.3. Efficiency of nanoparticles in transfection of pEGFP plasmid in the cultured HEK 293 cells

To examine the efficiency of alginate-chitosan, chitosan and alginate nanoparticles in transferring pEGFP plasmid to HEK 293 cells, these nanoparticles were incubated with the cells for 48 h and were compared to PolyFect transfection. Fourty eight hours post-transfection, the cells emitted the green fluorescence (Figure 4a) which was distinct from the non-transfected control cells (Figure 4b), under the immunofluorescent microscope.

Figure 4 shows the results of GFP expression in the HEK 293 cells. Flocytometric analysis of the transfected cells indicated 55%, 40%, 30.8% and 20.6% GFP expression for PolyFect, alginate nanoparticles, chitosan-alginate nanoparticles and chitosan nanoparticles, respectively. These results demonstrated the discrepancy of the transfection rate after 48 h incubation.

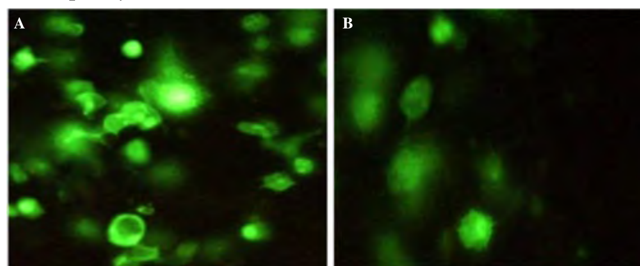

Figure 4. Fluorescent microscopy imaging of the transfected cells.

(a) The transfected HEK 293 cells showed the GFP expression for PolyFect. (b) GFP expression for alginate nanoparticles.

Transfection experiments were performed in PBS. Imaging was taken 48 h after the transfection.

3.4. Cytotoxicity assay of nanoparticles

The effect of the nanoparticle-plasmid complexes on the cell viability was determined using cytotoxicity assay. As illustrated in Figure 5, nanoparticles showed no toxicity toward HEK 293 cells after 4 h or 24 h.

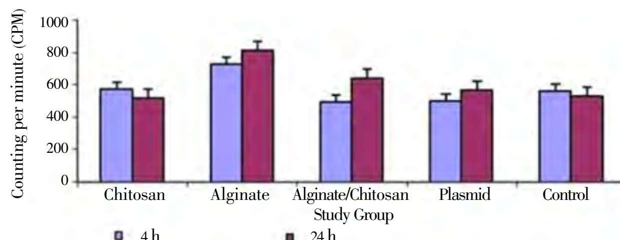

Figure 5. Cytotoxicity of test the compounds on HEK 293 cells at 4 and 24 h. The HEK 293 cells were cultured in 96-well plates and were exposed to 10 μg plasmid, alginate/chitosan nanoparticles, alginate and chitosan nanoparticles (26 μg/mL) and negative control (RPMI). All data are presented as mean of three different measurements±SE.

Moreover, the cells seemed to proliferate more quickly when treated with chitosan and alginate or both. Chitosan nanoparticles showed no significant differences with respect to cell viability, compared to the control. Although alginate nanoparticles showed significant increase in cell proliferation compared to the control, alginate-chitosan nanoparticles

showed no difference compared to the control after 4 h but had higher mean value of cell viability after 24 h ($P<0.05$).

4. Discussion

The basic concept underlying gene therapy is the use of DNA as a pharmaceutical agent for curing diseases. Nanoparticles formulated using chitosan or alginate, biodegradable polymers, have been widely investigated for targeted drug delivery[19,20].

The main purpose of this study was to determine the size and physicochemical properties of chitosan, chitosan-alginate and alginate nanoparticles. 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.

In this study, nanoparticles were prepared by pregel preparation method with alginate-chitosan ratio of 1, $CaCl_2$/alginate ratio of 0.2% and N/P ratio of 5 at pH 5.3. At pH 5.3, the majorities of amine groups of chitosan were protonated and were able to participate in electronic interactions with carboxyl group of alginate. Also the cationic characteristic of chitosan in this pH made it possible to combine with the plasmids[21]. Here, alginate can be cross-linked with the plasmids using polyvalent cations such as Ca^{2+} which results in producing the smallest nanoparticles in comparison to chitosan and alginate nanoparticles alone[22].

Moreover alginate-chitosan nanoparticles showed capacity of protecting DNA from enzymatic digestion. Also plasmids complexed with chitosan-alginate nanoparticles were protected from digestion, as demonstrated by the release of plasmid following inactivation of DNase I and degradation of the nanoparticles by chitosinase/lysozyme. Chitosan-DNA and alginate-DNA nanoparticles did not offer the same degree of protection from nucleases which prove that alginate-chitosan nanoparticles have more stability and afford more protection to DNA than either polymer alone.

The transfection rate of alginate nanoparticles was greater than alginate-chitosan and chitosan nanoparticles. Alginate-chitosan transfection was also greater than chitosan alone. The results also show that nanoparticles transfection and expression efficiency was less than PolyFect-plasmid. The reason for the greater transfection rate of alginate nanoparticles is due to alginate polymer properties. Alginate acts as a proton sponge which can increase endosomal release. Increasing the osmotic pressure of the endosomes is also caused by alginate degradation.

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[23-25].

The reason of greater efficiency in transfection of alginate-chitisan than chitosan nanoparticles was probably due to the limited ability of chitosan in controlling the release of plasmid because of its hydrophilic nature[25-27].

These results indicated that the presence of alginate with chitosan increases the proliferation of the cells when compared with the negative control (RPMI) which could be due to the influence of the mitochondrial activity of the cells[17], stimulating them to proliferate.

In conclusion, as an important finding, we demonstrated that the combination of particle surface, hydrophobicity size and zeta potential can influence on transfection efficiency and the cellular uptake of the nanoparticles. But on the other hand, alginate-chitosan nanoparticles were more stable than alginate nanoparticles. Also, alginate may reduce the electrostatic interactions between chitosan and the plasmid which can increase the plasmid release. Therefore our suitable candidate for gene delivery would be alginate-chitosan nanoparticles.

In vivo studies with a real vaccine are underway to investigate the efficiency of alginate-chitosan nanoparticles in a mucosal delivery system.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

To facilitate the delivery of genetic material, the use of appropriate carriers such as polymers is necessary. Chitosan and alginate are the most commonly studied polymers in recent years. Authors report preparation of three kinds of nanoparticles comprising chitosan, chitosan-alginate and alginate polymers and compare their size, cytotoxicity and cell transfection for a suitable candidate for gene delivery.

Research frontiers

Studies are being performed in order to determine which of these 3 kinds of nanoparticles can be smaller in size and whose cell transfection is better than the other, also if they can be harmful for the cells or not.

Related reports

There were other reports about preparing chitosan-alginate nanoparticles or chitosan nanoparticles demonstrating that they could be used for delivering plasmid to the cells or could be used in gene therapy, which was mentioned by the authors in their references. The results don't contrast with the results of almost similar papers, with the additional note that no report is available in respect of comparing the three mentioned nanoparticles at the same time.

Innovations & breakthroughs

As mentioned comparing chitosan, alginate and their combination is not already reported.

Applications

It can be used in cell transfection or oral drug delivery of chitosan/alginate nanoparticles.

Peer review

This study is interesting as the authors have compared the most important factors of nanoparticles on the most popular kinds of nanoparticles. Findings may help towards the production of more efficient gene delivery vehicles.

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