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Calcium-Alginate Nanoparticles Formed by Reverse Microemulsion as Gene Carriers

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Summary: Natural biopolymers are widely used in the field of drug and gene delivery. In this study, alginate nanoparticles were prepared using water-in-oil microemulsion as a template followed by calcium crosslinking of guluronic acid units of alginate polymer. After collected by ultracentrifugation, alginate nanoparticles were analyzed by electron microscopy to obtain the size and morphology which were varied with the ratio of water, oil, and surfactant used. To examine the potency of Ca-alginate nanoparticles as carriers for gene delivery, GFP-encoding plasmids were encapsulated in these nanoparticles to investigate the degree of endocytosis by NIH 3T3 cells and ensuing transfection rate. Our results showed that Ca-alginate nanoparticles with an average size around 80 nm in diameter are very efficient gene carriers, in comparison with plasmid DNA condensed by polyethyleneimine (PEI).

Keywords: AOT; calcium-alginate nanoparticle; gene delivery; polyethyleneimine; reverse microemulsion

Introduction

It has been shown that the potential risks (e.g., immunogenicity and oncogenicity) of using viral vectors for gene therapy are obstacles for clinical use even though virus-mediated gene delivery offers high transduction efficiency. As a result, much attention has been focused on the design of synthetic cationic vectors for nucleic acid condensation and delivery. Despite the promise of developing clinically useful nonviral vectors, most nonviral gene carriers provide less efficient gene transfer, especially in escape from endosomal vesicles. Although quite a few endosomolytic chemicals (e.g., polyethyleneimine [1], chloroquine [2], and cationic lipid [3]) have been used to facilitate endosomal escape of plasmid DNA, cytotoxicity induced by these agents is a major obstacle need to tackle with. In this study, we propose to use Ca-alginate nanoparticles as the gene delivery vehicle since it is biocompatible and biodegradable. Moreover, once internalized via endocytosis pathways, they can undergo quick erosion and elicit osmotic swelling, hence facilitating endosomal escape of gene to the cytoplasm.

In order to make alginate-based nanoparticles, we employed self-assembly microemulsion as the template. The water-in-oil (w/o) microemulsion system is thermodynamically stable; typically the composition is 10, 5, and 85% by volume of the water, surfactant, and oil, respectively. Water-in-oil microemulsions generally contain nanometer-sized water droplets stabilized by a curved surfactant monolayer. To date, a good range of different microemulsion systems has been used, and polymerization of the continuous, dispersed and surfactant phase has been attempted.[4,5] Daubresse et al. have described an interesting approach for immobilizing enzymes in nanoparticles prepared from microemulsions.[6] Both nonbiodegradable poly(acrylamide) and biodegradable poly(acryldextran) nanoparticles were prepared using w/o microemulsions stabilized by a mixture of Aerosol-OT (AOT; sodium bis(2-ethylhexyl) sulfosuccinate) and Brij 30. Addition of the enzyme alkaline phosphatase to the reaction mixture caused the enzyme to become entrapped in the nanoparticles. The resulting particles were typically between 25 and 50 nm, with a polydispersity of ~ 20%. However, to the best of our knowledge, no study has yet demonstrated the capability of making nanometer-scale Ca-alginate particles using water-in-oil microemulsion as the template.

For the reverse microemulsion developed here, toluene was used as the oil phase, AOT was the surfactant, and 0.5% sodium alginate (Na-alginate) solution was the aqueous phase. AOT is one of the most investigated anionic surfactants. Because of its double-tailed nature, the AOT molecule is almost structurally balanced with respect to its hydrophilic and hydrophobic parts and is one of the few ionic surfactants that can form microemulsions without the addition of a co-surfactant.[7-10] After washing AOT from the reverse microemulsion template by toluene, the structure of Ca-alginate nanoparticles has a core of Na-alginate and an enclosed membrane formed by gelation of Na-alginate using calcium chloride. For the encapsulation of plasmid DNA, plasmids encoded with enhanced green fluorescent protein (eGFP) were pre-mixed with sodium alginate solution and then added dropwise into AOT/toluene mixture. The formed DNA/alginate nanocontainers were gelled by the addition of calcium chloride. The formed DNA/alginate nanoparticles were cultured with NIH 3T3 cells and led to 50% cells with the expression of eGFP post 48-h incubation, which is comparable to the result done by PEI/DNA complexes.

Materials and Methods

Preparation of Ca-alginate Nanoparticles

First, 0.25 g of AOT (sodium bis(2-ethylhexyl) sulfosuccinate, Sigma) was dissolved into a vial pre-filled with 13.11 g of toluene. Then, 0.5% sodium alginate solution (medium viscosity; viscosity of 2% solution is approximately 3,500 cps at 25°C, Sigma) was added dropwise into the vial with a micropipette. Various phase appearance (from transparent to turbid) was obtained during the process of adding alginate solution. For each distinct phase, the mixture was vortexed and waited for 10 minutes prior to the addition of 2 mL of filtered calcium chloride solution (2% by weight) for obtaining alginate-based nanodroplets by crosslinking. After carefully washed with acetone and deionized water to remove all residual AOT and toluene, the vial was centrifuged (33,000 ×g) for 30 min and a small white pellet of material was obtained. Finally, the prepared nanoparticles were re-suspended with deionized water. The procedure of encapsulating plasmid DNA into Ca-alginate nanoparticles was the same as the one just described above, except pDNA was pre-mixed with 0.5% sodium alginate solution.

Characterization of Ca-alginate Nanoparticles

The size and morphology of prepared Ca-alginate nanoparticles were analyzed by transmission electron microscopy (model 420, Philips, Eindhoven, Netherlands) and scanning electron microscopy (model S-570, Hitachi, Tokyo, Japan). For TEM images, aqueous solution of the nanoparticles was dropped onto a copper grid (200 mesh) supporting a thin film of amorphous carbon. The excess liquid was swept away with a filter paper, and the grid was dried in air. For SEM images, a drop of aqueous solution containing Ca-alginate nanoparticles was placed on a specimen stub and coated with gold-palladium by sputtering for 2 minutes with 50 mA (K-550X, Emitech LTD, Kent, England).

Transfection of NIH 3T3 Cells

NIH 3T3 cells in the exponential growth phase were detached with 1X EDTA-trypsin (Irvine Scientific, Santa Ana, CA) from a culture dish (Falcon, Franklin Lakes, NJ). 5×10^4 of NIH 3T3 cells were inoculated on a plastic petri dish (35 mm in diameter) with 2 mL of high-glucose Dulbecco's modified Eagle medium (DMEM, Mediatech Inc., VA) supplemented with 10% fetal bovine serum (FBS, Irvine Scientific). After 24-h incubation, Ca-alginate nanoparticles encapsulated with 2 μg plasmid DNA encoding eGFP (pIRES2-EGFP vector, Clontech, Palo Alto, CA) were suspended in 2 mL of growth medium and layered on top of

the NIH 3T3 cells which were attached on the bottom of the culture dish. NIH 3T3 cells exposed to pDNA encapsulated Ca-alginate nanoparticles were incubated for transfection. All cell cultures were performed at 37°C and balanced with 5% CO_2 in a 100% humidified incubator. For comparison, transfection of NIH 3T3 cells was also performed using PEI/DNA complexes (MW of PEI = 750 kDa; N/P ratio = 6, where N is the amount of amino nitrogens of PEI and P stands for the amount of phosphate groups of the plasmid DNA).

Results and Discussion

Determination of Reverse Microemulsion Domain

In order to obtain particles with a diameter close or less than 100 nm, water soluble sodium alginate was introduced into diluted water-in-oil microemulsion stabilized by AOT surfactant. Since sodium alginate is insoluble in the oil (toluene), the alginate polymer is confined within the aqueous nanophase. The phase stability of the resulting microemulsion is significantly affected by the amount of added alginate solution. As shown in Figure 1, point A (toluene:alginate solution:AOT = 86.1:0.6:13.3 by weight) in the phase diagram domain indicates the region of transparent microemulsion. Upon addition of sufficient 0.5% alginate solution, the mixture was transformed into translucent (point B) then milk-like appearance (point C).
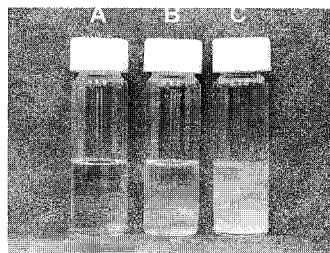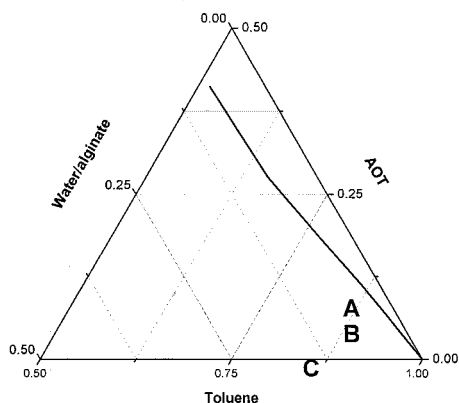

Figure 1. Phase diagram for toluene/alginate solution/AOT (micro)emulsion. Points A (toluene/alginate solution/AOT = 86.1:0.6:13.3 by weight) and C (toluene/alginate solution/AOT = 81:10.8:8.2 by weight) refer to compositions used for the preparation of alginate microemulsion and emulsion. Point A corresponds to the w/o microemulsion region, while, point C is in the emulsion region. Point B (toluene/alginate solution/AOT = 85.7:1.8:12.5 by weight) is located on the boundary line distinguishing the domains of microemulsion and emulsion.

Size and Morphology of Ca-alginate Nanoparticles

The alginate microemulsion at point A in the phase diagram was further mixed with calcium chloride solution to form Ca-alginate nanoparticles. Figure 2 shows the TEM and SEM images of Ca-alginate nanoparticles obtained from the template of microemulsion, boundary, and emulsion, respectively. Figure 2 (A) illustrates separated and spherical nanoparticles with the size ranged from 55 nm to 100 nm. In Figure 2 (B), necklace-like Ca-alginate nanoparticles were identified. More specifically, the size of each single nanoparticle was bigger than that of nanoparticles formed by the microemulsion template. We speculate that alginate biopolymer may interfere with the interdroplet correlations, particularly if it is too large to fit into a single droplet and may thus require a cluster of several droplets for accommodation. To be exact, the biopolymer is covered with a linear array of discrete droplets so as to make up a necklace. In Figure 2 (C), prepared Ca-alginate particles further aggregate with rough morphology. For polymer chains too long to be accommodated in a single particle, necklaces may be formed as shown above. In order to prevent potential aggregation of alginate nanoparticles in the process of gelation within w/o microemulsion template, alginate polysaccharides probably need to be trimmed down to small molecular weights.
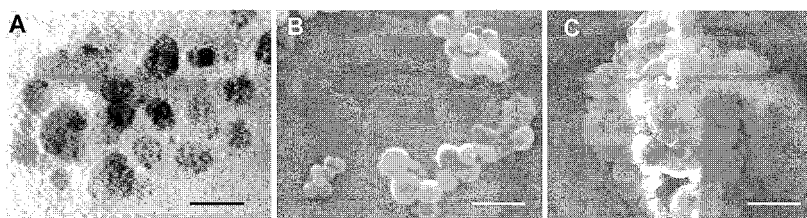

Figure 2. Images of Ca-alginate particles. a) TEM image nanoparticles formed from point A in the microemulsion zone of the phase diagram given in Figure 1 (scale bar = 100 nm; b) SEM image of nanoparticles obtained from point B in the boundary zone (scale bar = 300 nm); c) SEM image of particle aggregates by calcium crosslinking of point C in the emulsion zone (scale bar = 1 μm).

Transfection Rate Enhanced by Ca-Alginate Nanoparticles Encapsulated with pDNA

To examine the efficacy of Ca-alginate nanoparticles in facilitating endosomal escape of pDNA, NIH 3T3 cells were incubated with nanoparticles obtained from the microemulsion template (i.e., point A of Figure 1) for 24, 48 and 72 h, respectively. Percentages of eGFP-expressing NIH 3T3 cells were determined by both bright-field and fluorescent microscopy

(see Figure 3-I). For comparison, transfection of NIH 3T3 cells was also performed using PEI/DNA complex (see Figure 3-II) which has been known for its capability of enhancing pDNA release from endosomes. As shown in Figure 3, the discrepancy of transfection rate between using PEI/DNA complexes (53%) and pDNA encapsulation in Ca-alginate nanoparticles (8.5%) was prominent after 24-h incubation. However, after 48-h incubation, the transfection rate using Ca-alginate nanoparticles encapsulated with pDNA was greatly increased up to 48%, in comparison with the one using PEI/DNA complexes (55%). The reason causing such dramatic enhancement of transfection rate is probably because endosomal swelling, elicited by Ca-alginate nanoparticles, reaches its peak after 48-h incubation, hence expediting the release of encapsulated pDNA into cytosol. Transfection rates were also quantitatively determined by measuring the intensity of eGFP expression in cells using a fluorimetric microplate reader with an excitation wavelength of 472 nm and emission wavelength of 512 nm (shown in Figure 4). The trends of this result were consistent with the one given in Figure 3.
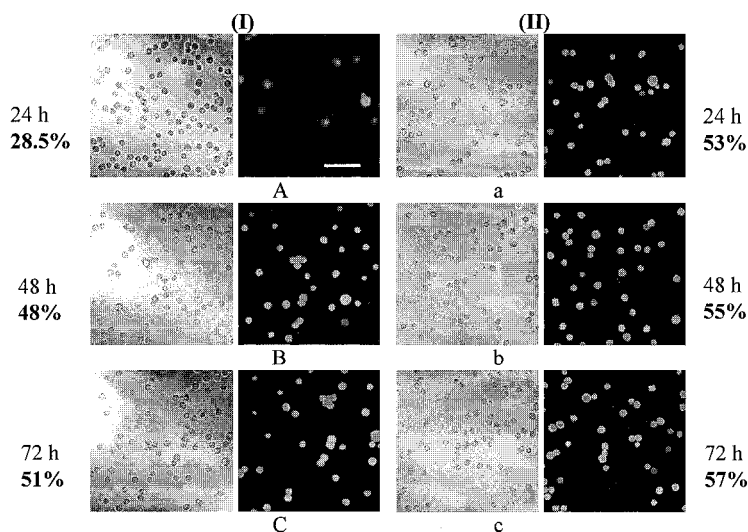

Figure 3. Photomicrographical images of eGFP-expressing NIH 3T3 cells under both bright-field and fluorescent microscopy after been incubated with (I) pDNA encapsulated in Ca-alginate nanoparticles and (II) pDNA condensed with PEI for 24 h (A, a), 48 h (B, b), and 72 h (C, c), respectively. These images represent one set of triplicate experimental data. Note the cells were suspended by trypsinization before the images were taken. Scale bar = 50 μm.
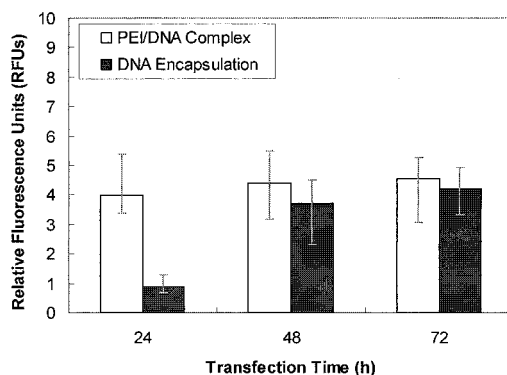
Figure 4. Transfection efficiency of NIH 3T3 cells treated separately with PEI/DNA complex and Ca-alginate nanoparticles encapsulated with pDNA for various periods of time. The relative fluorescence units of eGFP-expressing NIH 3T3 cells were detected by a fluorimetric microplate reader. All the experimental data were obtained in triplicate.

Conclusion

The size of Ca-alginate nanoparticles was significantly modulated by the ratio of toluene, AOT, and sodium alginate solution. Ca-alginate nanoparticles with an average size of 80 nm were able to encapsulate plasmid DNA and ferry the gene into non-phagocytic cells via endocytosis pathway. It seems that the alginate-based gene carriers can assist DNA escape within 1-2 day timeframe from the endosomal lumen into the cytoplasmic space, leading to high transfection efficiency.

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