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Calcium Alginate Microparticles As a Non-Condensing DNA Delivery and Transfection System for Macrophages

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Introduction

Inflammation is a defense mechanism adopted by the body in response to the variety of stimuli, including pathogens, injury, and autoimmune responses.[1,2] The primary functions of macrophages in inflammation include antigen presentation, phagocytosis, and modulation of the immune response through production of various cytokines and growth factors.[1,3] In case of inflammation caused by exposure to pathogens, the process of phagocytosis is mediated by specific receptors expressed on the surface of macrophages and other immune cells. Additionally, the attachment of antibodies and complement fragments, by a process called *opsonization*, to the microbes greatly enhances the phagocytic ability of macrophages.[1] The classical macrophage activation state is characterized by killing of intracellular pathogens and tumor resistance and can be induced by interferon-γ (IFN-γ) alone or in conjunction with microbial products such as Lipopolysaccharide (LPS) or cytokine, such as Tumor-Necrosis Factor alpha (TNF-α). The alternative state can be induced by cytokines, such as IL-4 and IL-13, and mainly results in anti-inflammatory responses and resolution of injury. Activation of macrophage via the classical pathway is marked by high antigen presentation capacity, high IL-12, IL-23, nitric oxide (NO), and reactive oxygen species production. On the other hand, alternate activation stage is characterized by an increase in the IL-10 and IL-1ra cytokines, mannose and scavenger receptors, arginase production, and decrease in the production of inducible nitric oxide synthase enzyme.[4-6]

Therefore, it is evident that macrophage activation will have a significant impact on the progression of pathologic conditions, such as growth and spread of malignant tumors, sepsis, chronic inflammation in rheumatoid arthritis, lysosomal storage disease, atherosclerosis, and major infections including HIV/AIDS and tuberculosis. Therefore, development of therapeutic delivery strategies aimed at macrophage-specific processes has potential for treating a variety of conditions.

Alginate is a random block copolymer made up of (1 → 4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues and occurs in nature as a structural component of marine brown algae (*Phaeophyceae*), where it comprises 40% of the dry matter. It also occurs as a component of capsular polysaccharide in soil bacteria.[7] Alginate is considered by the United States Food and Drug Administration (US FDA) as a *"generally regarded as safe"* or GRAS material and has found applications in various industries including food, pharmaceutical, and cosmetic industries.[8] Many of the applications of alginate rely on the ability of the polymer to form cross-linked hydrogels in the presence of di- and trivalent cations, such as calcium ions (Ca^{2+}).

In order to form Ca^{2+} ions cross-linked alginate particles, the electrolyte has to be introduced in a very controlled fashion using the diffusion (external gelation) method. In this process, Ca^{2+} ions are allowed to diffuse from a large outer reservoir into alginate solution. This technique exhibits rapid gelation kinetics and is suitable for immobilization purposes where each drop of alginate forms a single gel bead with entrapped bioactive agent. The formulation parameters, such as sodium alginate molecular weight and concentration, stirring

conditions, and rate of Ca^{2+} ions addition can be further optimized to form particles in the nanometer range.[10]

Ca^{2+}-alginate hydrogel particles also have been used as a non-viral gene delivery system because of their biocompatibility and ability to protect the plasmid DNA from enzymatic and pH-induced degradation. Douglas et al[11] reported that inclusion of alginate to chitosan-based nanoparticles improved the transfection efficiency of encapsulated plasmid DNA by four-fold as compared with control. Also, it was shown via cell viability assay, gel-retardation assay, and transfection studies that an alginate-chitosan/DNA based system exhibited lower toxicity, protected the DNA from DNase I degradation, which was not achieved by chitosan based nanoparticles alone, and improved the transfection efficiency in the 293T cell. Additionally, at 48 hours post-administration, this group was able to show that the transfection efficiency of alginate-chitosan nanoparticles was as high as Lipofectamine™. In the same context, Jiang, et al[12] aimed at improving the transfection efficiency and lower the cytotoxicity of poly(ethyleneimine) (PEI)/plasmid DNA complex by coating with anionic biodegradable polymer, alginate. The group reported that coating with alginate improved the transfection efficiency to the C3 cells by 10-30 folds in comparison to the non-coated PEI/DNA complex. In addition, the alginate/PEI/DNA complex showed a reduced erythrocyte aggregation and lower cytotoxicity profile to C3 cells in comparison to PEI/DNA complex alone.

Previously, non-condensing polymeric systems that can physically encapsulate plasmid DNA, such as type B gelatin, have been shown to afford more efficient and sustained transgene expression relative to cationic lipids and polymers.[13,14] Type B gelatin-based nanoparticles have been utilized for systemic and oral gene therapy using a variety of reporter and therapeutic plasmid DNA. We have postulated that the non-condensing system can retain the supercoiled structure of the plasmid DNA and allows for more efficient nuclear entry in non-dividing cells. Most importantly, these constructs are significantly less toxic to cells as compared to cationic lipid and polymeric transfection reagents. To further the application of non-condensing polymers for gene therapy, in this study we have developed Ca^{2+} alginate microparticles with encapsulated reporter plasmid DNA expressing GFP (i.e., EGFP-N1) and have evaluated the delivery efficiency and transgene expression using J774A.1 adherent macrophage cell line.

Materials and Methods
Materials
High viscosity grade sodium alginate was purchased from Protanal (Norway) and calcium chloride dihydrate was purchased from Sigma Aldrich (St. Louis, Missouri, USA), and were dissolved in de-ionized distilled water. Plasmid DNA expressing enhanced green fluorescence protein (i.e., EGFP-N1, 4.7 kb) was purchased from Clontech and amplified and purified by Elim Biopharmaceuticals (Hayward, Californiam USA). Rhodamine-B labeled dextran (Mol Wt. 70 kDa), the supercoiled DNA ladder (2-16 kb), were purchased from Invitrogen (Carlsbad, California, USA). Alginate lyase enzyme

was purchased from Sigma Aldrich (St. Louis, Missouri, USA). Pluronics® F-108 was purchased from BASF chemicals (Mount Olive, New Jersey, USA).

Preparation of DNA-Encapsulated Alginate Microparticles
A stock solution with 1% (w/v) medium viscosity sodium alginate (Protanal® LF 20/200) solution was prepared. Similarly, a stock solution of 0.5M calcium chloride dihydrate (M.W. 147.02) (Fisher) was made. A 3 ml of sodium-alginate solution was filled into a 5 ml syringe fitted with a 30G1/2-inch needle. The alginate solution was added drop-wise into calcium chloride solution (27 ml) while stirring at 2,400 rpm using a 4-blade lab stirrer. Furthermore, these formulations were stabilized by adding Pluronics® F-108 (0.1% w/w of alginate) to the sodium-alginate solution prior to cross-linking with calcium. Pluronics® F-108 (PEO) is a copolymer which is made up of 56 residues of propylene oxide (PO) and 122 residues of ethylene oxide (EO). The resulting particle suspension was centrifuged at 10,000 rpm for 35 minutes. The pellet was washed twice with deionized water. 5 ml of de-ionized water was added to the pellet and to the resulting suspension, 0.1% (w/w) mannitol (Acros Organics) was added as a cryoprotectant. The sample was then freeze-dried at -80°C and later lyophilized to get the particle cake.

Characterization of the Microparticle Formulations
Particle Size, Surface Charge, and Morphological Analyses: The particle size and surface charge (zeta potential) of the blank and DNA incorporated particles were measured using the Coulter Counter Coulter Particle Size Analyzer at Massachusetts Institute of Technology (MIT), Boston, Massachusetts, USA. The sample obtained after lyophilizing the freeze-dried formulation was analyzed by Scanning Electron Microscope (SEM) for surface morphology and size. The sample was mounted on an aluminum sample mount and sputter-coated with a gold-palladium alloy to minimize surface charging. SEM was performed using Hitachi Instruments' S-4800 environmental scanning electron microscope (San Jose, California, USA) at an accelerating voltage of 3 kV.

Determination of Plasmid DNA Loading and Stability: 20 µg of EGFP-N1 plasmid DNA dissolved in aqueous solution was added to alginate solution prior to cross-linking with calcium. Plasmid DNA encapsulation efficiency was measured using PicoGreen® dsDNA fluorescence assay (Invitrogen) following digestion of the polymer matrix of the microspheres with the enzyme alginate lyase (1 mg/ml) for 24 hours in phosphate-buffered saline (PBS, pH 7.4) at 37°C. Following centrifugation at 13,000 rpm for 30 minutes, the supernatant was collected and the released DNA was quantified using PicoGreen® fluorescence reagent with a Bio-Tek Synergy® HT (Winooski, Vermont, USA) microplate reader. The stability of encapsulated plasmid DNA, due to processing conditions, was assessed using agarose gel electrophoresis. Following extraction of the DNA from the freeze-dried sample of

nanoparticles using 1mg/ml alginate lyase and precipitation with ethanol, a sample was run on 1.2% pre-casted ethidium bromide-stained agarose gels (Invitrogen). Control lanes had 2-16 kb DNA ladder and the naked plasmid DNA sample. Following the agarose gel electrophoresis, the ethidium bromide labeled DNA bands were visualized with a Kodak FX imager (Carestream, Rochester, New York, USA).

Macrophage-Specific Uptake and Cytotoxicity Analyses

Cell Culture Conditions: J774A.1 adherent murine macrophage cell line was obtained from American Type Culture Collection (ATCC Manassas, Virginia, USA) and grown in T75 culture flask at 37°C and 5% CO2 using Dulbecco's modified Eagle medium (DMEM Cellgro®, Mediatech Inc., Manassas, Virginia, USA) modified with 10% fetal bovine serum (FBS Gemini Bio-Products, West Sacramento, California, USA) and combination penicillin/streptomycin antibiotics. Cells were allowed to divide until they reached desired density. Cell count was measured by placing 20 μL of the cell suspension mixture on a heamocytometer slide and the cell viability studies were performed using Trypan blue dye exclusion assay.

Macrophage-Specific Particle Uptake and Cellular Internalization: In order to evaluate the uptake and cellular internalization of calcium-alginate microspheres, rhodamine-B dextran was encapsulated at 1% (w/w) concentration using a similar procedure as described above for plasmid DNA. Particles were incubated with 20,000 J774A.1 macrophages, plated on glass cover-slips, in a 6-well microplate in the presence of DMEM supplemented with 10% FBS. The cells were treated with microspheres in a time-dependent fashion from 1-6 hours; however, only 6 hour time point has been shown here. After particle treatment, the cells were placed on the glass cover slips, placed in the 6-well microplate were removed and rinsed with sterile PBS, and inverted on a clean slide for qualitative analysis of uptake and cellular internalization using fluorescence microscopy. Bright field and fluorescence images were acquired with a BX51-TRF Olympus (Center Valley, Pennsylvania, USA) inverted microscope at 20× and 40× original magnifications.

Cytotoxicity Analysis Using MTT Reagent: Blank and plasmid DNA-encapsulated alginate particles were incubated with 10,000 J774A.1 macrophages in 96-well microplates for cytoxicity analysis in the presence of DMEM supplemented with 10% FBS conditions. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) reagent (MTT Promega, Madison, Wisconsin, USA) that is converted to water-soluble formazan derivative by viable cells was used to assess cytotoxicity of the formulations. Untreated cells were used as negative control, while poly(ethyleneimine) (PEI; Mol. wt. 10 kDa), a known cytotoxic cationic polymer at a concentration of (1 mg/ml), was used as a positive control. A known amount of micro-particles sample with and without encapsulated plasmid DNA (20 μg) were suspended in 200 μL of culture media and incubated with the cells for 6 hours.

Following a washing step with sterile PBS, the wells were treated with MTT reagent and the stop mix is then added to the culture wells to solubilize the formazan product, and the absorbance of the chromogenic formazan product in viable cells was measured at 570 nm BioTek Synergy HT microplate reader. Percent cell viability was calculated from the absorbance values relative to those of untreated cells. The samples were tested with n=8 replicates.

EGFP-N1 Plasmid DNA Transfection Studies

Calcium alginate microspheres with encapsulated plasmid DNA expressing reporter GFP (i.e., EGFP-N1) were added to J774A.1 macrophages in a 6-well micro-plate, in the presence of DMEM supplemented with 10% FBS, at a dose equivalent to 20 μg of DNA per 200,000 cells. Naked plasmid DNA and DNA-complexed with the cationic lipid transfection reagent Lipofectin® (Invitrogen, Carlsbad, California, USA) were used as controls. Following 6 hours of incubation, the wells were rinsed with sterile PBS to remove excess particles and 2 mL of FBS supplemented DMEM was added. Periodically, starting from 24 hours to 96 hours post-administration, quantitative analysis of transgene expression was carried out with a GFP-specific enzyme-linked immunosorbent assay (ELISA). Transfected cells were harvested, lysed, and the cell extract was used for determination of GFP concentrations relative to the total intracellular protein concentration obtained using a BCA Assay (Thermo Scientific-Pierce, Rockford, Illinois, USA). A 96-well microplate was coated with 100 μL of anti-GFP mouse monoclonal antibody (Novus Biologicals, Littleton, Colorado, USA) diluted at a concentration of 1:2400 and incubated for 2 hours at 25°C. The antibody-coated microplate was then washed 5-times with PBS-T washing buffer (Sigma-Aldrich, St. Louis, Missouri, USA) and then blocked with 200 μL of blocking buffer (Thermo Scientific-Pierce, Rockford, Illinois, USA) for 2 hours at room temperature. The microplate was again washed 5 times and 100 μL of cell lysate was added and incubated at 4°C overnight. Following extensive rinsing with the washing buffer, 100 μL polyclonal secondary antibody conjugated to alkaline phosphatase (Novus Biologicals, Littleton, Colorado, USA) was added and incubated for 1 hour at room temperature. Lastly, 100 μL of the substrate was added to the wells and the chromogen was measured at 409 nm using the microplate reader. A calibration curve was constructed using GFP (BioVision, Mountain View, California, USA) and the levels of transfected GFP in macrophages were calculated as ng per mg of total cellular protein.

Qualitative analysis of GFP expression as a function of time after incubation of J774A.1 macrophages with EGFP-N1 plasmid DNA-encapsulated alginate particles was determined by fluorescence microscopy. Naked plasmid DNA and DNA-complexed with Lipofectin® were used as controls, Followed by treatment with 20 μg equivalent dose of DNA per 200,000 cells for 6 hours in a 6-well microplate, in the presence of DMEM supplemented with 10% FBS, having glass cover slips in each well, and the cells were incubated at 37°C. At pre-determined time intervals from 24 hours to 96 hours post-treatment, the cover slips were removed, rinsed with
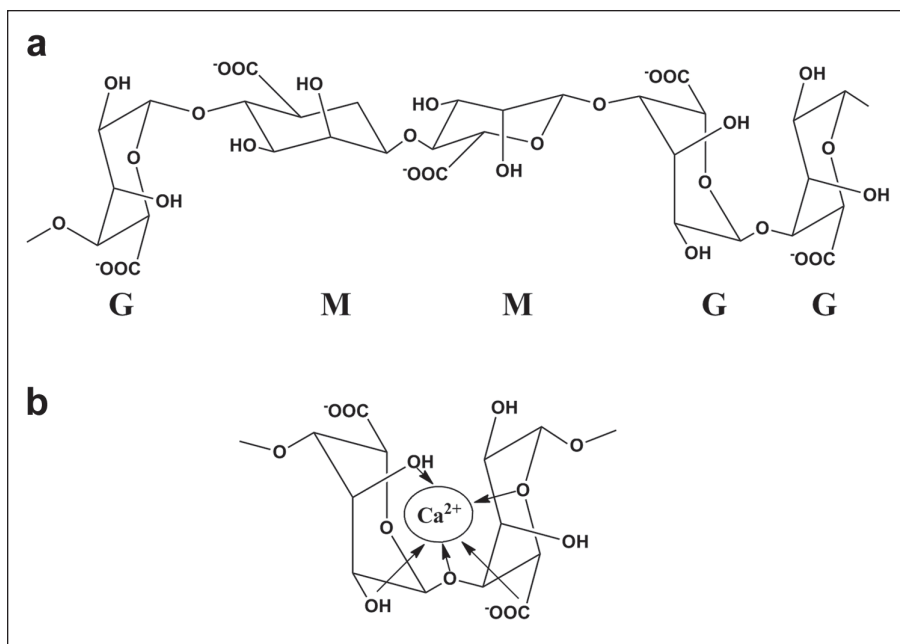
Figure 1. (a) The chemical structure of alginate showing two repeating monomer units – mannuronic acid (M) and guluronic acid (G) and (b) ionic gelation with divalent cations, such as calcium ions, leads to the formation of "egg box" structure.

sterile PBS, and placed on a glass slide. GFP expression in the cells was visualized by fluorescence microscopy using an inverted Olympus microscope.

Statistical Data Analysis

Statistical significance of results was determined using one-way ANOVA and Tukey's Multiple Comparison Test with a 95% confidence interval ($p < 0.05$).

Results
Preparations and Characterization of Calcium Alginate Microparticles

As a GRAS material, alginate has been used in a variety of applications. In this study, we have prepared Ca^{2+} ion cross-linked alginate microparticles for macrophage-specific gene delivery and transfection. Figure 1 shows the chemical structure of the repeat G and M units of alginate, the "egg box" model, that is used to describe the Ca^{2+} ion cross-linked alginate matrix.

Table A shows the particle size and surface charge of

Formulation	Hydrodynamic Diameter (μm)	Zeta Potential (mV)
Blank PEO-Modified Alginate Microparticles	1.02 ± 0.23*	-8.60 ± 1.60
Plasmid DNA-Encapsulated PEO-Modified Alginate Microparticles	0.87 ± .07	-13.5 ± 2.30
*Mean ± S.D. (n = 3)		

Table A. Particle size and surface charge analyses of blank and plasmid DNA-encapsulated calcium ion-crosslinked poly(ethylene glycol) (PEO)-modified alginate microspheres.

both the blank and DNA encapsulated optimized calcium alginate microparticles modified with 0.1% (w/w) F-108 Pluronics®. The particle size of the optimized DNA encapsulated formulation was found to be ~800 nm and surface charge was found to be on average -13.5 mV, whereas the particle size of the blank formulation was ~1 μm and the surface charge was -8.6 mV. Furthermore, the SEM results in Figure 2 confirmed that the optimized DNA-loaded formulation was found to be smooth and spherical in shape with an average particle size of about 1 μm.

Figure 3 shows the stability of encapsulated plasmid DNA due to processing conditions using agarose gel electrophoresis. Lane 1 is 2-16 kb super-coiled double stranded DNA ladder, lane 2 is precipitated naked EGFP-N1 plasmid DNA showing open and circular bands, lane 3 shows the plasmid EGFP-N1 DNA extracted from the supernatant of calcium-alginate microspheres treated with 1mg/ml of alginate lyase for 24 hours at 37°C. Lane 4 shows the pellet obtained after centrifuging the particles treated with alginate lyase. As evident, no plasmid DNA bands were observed in this lane indicating that alginate lyase treatment for 24 hours was sufficient enough to completely degrade the polymer and as a result, the total amount of encapsulated plasmid DNA was released and collected in the supernatant. Overall, these results show that the plasmid DNA can be efficiently protected in the micro-particle matrix.

In addition, the plasmid DNA loading studies using pico-green analysis revealed that the plasmid loading efficiency was around 65%.

Microparticle Uptake and Cytotoxicity in Macrophages

Figure 4 represents the fluorescence images obtained for control (untreated cells) and rhodamine-labeled micro-particles at 20× and 40× magnifications. The studies were conducted in a time-dependent fashion; however, the fluorescence images for only a 6 hour time point have been shown here as appre-
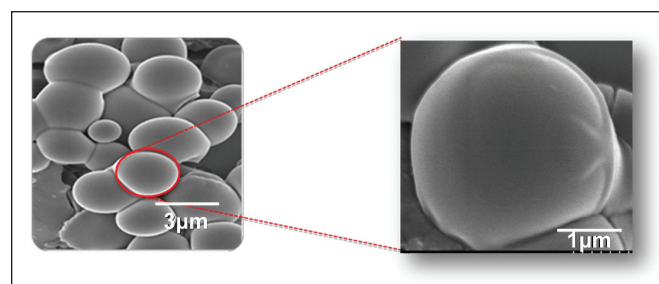

Figure 2. Scanning electron microscopy image shows spherical uniformly-sized plasmid DNA-encapsulated calcium alginate microspheres. Higher magnification image of one of the microspheres shows smooth surface morphology.
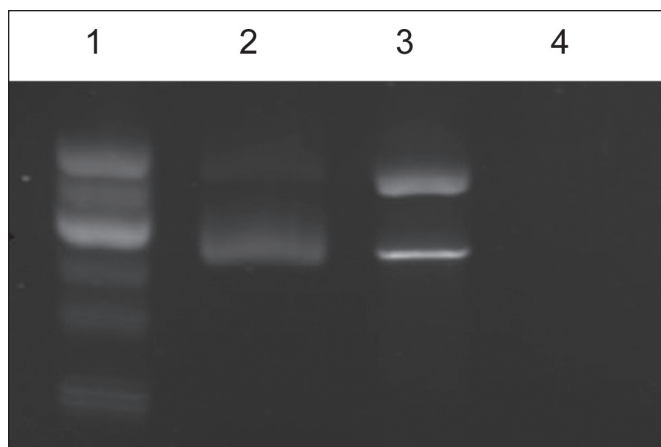
Figure 3. Evaluation of plasmid DNA stability by agarose gel electrophoresis. Lane 1 is 2-16 kB DNA ladder, lane 2 is naked EGFP-N1 plasmid DNA after precipitation showing two bands corresponding to open circular and supercoiled DNA, lane 3 is EGFP-N1 plasmid DNA extracted from the microsphere formulation after incubation with 1 mg/ml alginate lyase for 24 hours, and lane 4 is the pellet obtained after centrifuging the formulation treated with 1 mg/ml alginate lyase.

ciable amount of signal was observed only at this point. The particles were suspended in the complete DMEM media and incubated with the cells for 6 hours and subsequently cells were viewed under fluorescence microscope. These images confirmed that alginate based microspheres were efficiently phagocytosed by the J774A.1 macrophages at 6 hours post-administration. Based on this data, it was decided that for subsequent toxicity and GFP transfection analysis, particles will be incubated with the cells for 6 hours.

In order to assess potential cytotoxicity, if any, with the control and EGFP-N1 plasmid DNA-encapsulated alginate particles, the formulations were incubated with J774A.1 macrophages. In viable cells, the enzymes convert the yellow MTT reagent, in the presence of phenazine methosulfate, to a purple-colored formazan product that has an absorbance maximum at 570 nm. The cell viability results, as shown in Figure 5, confirm that neither the blank nor DNA-loaded formulations induced any significant cytotoxicity. The cell viability was maintained at approximately 100% in both cases. In comparison, PEI-treated cells, at a concentration of 1 mg/ml, caused significant cell cytotoxicity and cell viability was significantly reduced to about 35% after 6 hours of incubation.

Quantitative and Qualitative Transfection Analyses

A GFP-specific ELISA (Figure 6) was used for quantitative determination of the transgene expression in J774A.1 macrophages upon treatment with control and DNA-loaded calcium-alginate microparticles. The results show the intracellular GFP per total protein concentrations as a function of time ranging from 24 hours to 96 hours post-administration. On average, highest GFP expression (i.e., 0.65 ng/mg) was observed at 24 hours post-administration. In comparison, Lipofectin® and naked plasmid showed on average transgene
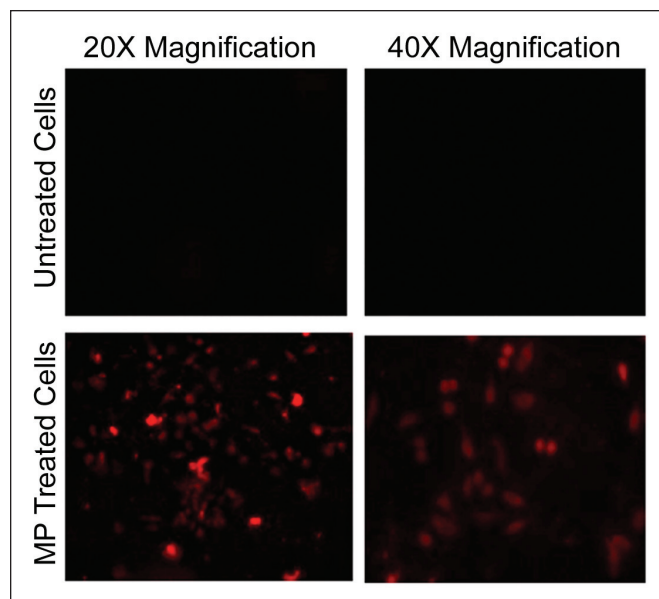

Figure 4. Cellular uptake and intracellular localization of rhodamine-labeled calcium alginate microsphere in J774A-1 macrophages. Top panel images are of untreated cells at $20 \times$ and at $40 \times$ magnification, whereas bottom panel images are of cells treated with rhodamine-labeled microspheres at $20 \times$ magnification and at $40 \times$ magnification.

expression of 0.41 ng/mg ($p < 0.001$) and 0.05 ng/mg ($p < 0.0001$), respectively, at this time point. For the subsequent time points, the GFP levels still remained significantly higher in the calcium-alginate microsphere treatment group as compared to positive controls including Lipofectin® and naked plasmid DNA.

Figure 7 shows the qualitative GFP expression analysis using fluorescence microscopy images of J774A.1 macrophages transfected with EGFP-N1 plasmid DNA in the control and alginate microparticle formulations. The GFP expression
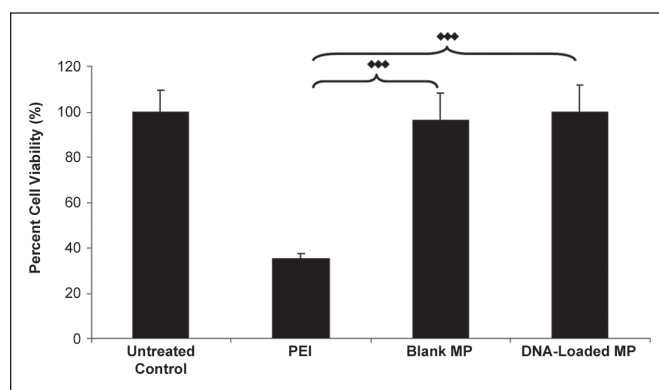

Figure 5. Cytotoxicity analysis of calcium alginate microspheres in J774A-1 macrophages evaluated by MTT (formazan) assay. The cytotoxicity of the plasmid DNA loaded formulation in macrophages was compared to untreated cells. Poly(ethyleneimine) (PEI, Mol. wt. 10 kDa) served as a positive control. The cell viability of the / untreated cells was considered 100% and the values obtained in the rest of the treatment groups were normalized to control values and presented as percent viability. The values reported are mean ± SD. (n = 8). Statistical significance of results was determined using one-way ANOVA and Tukey's Multiple Comparison Test with a 95% confidence interval ($p < 0.05$).
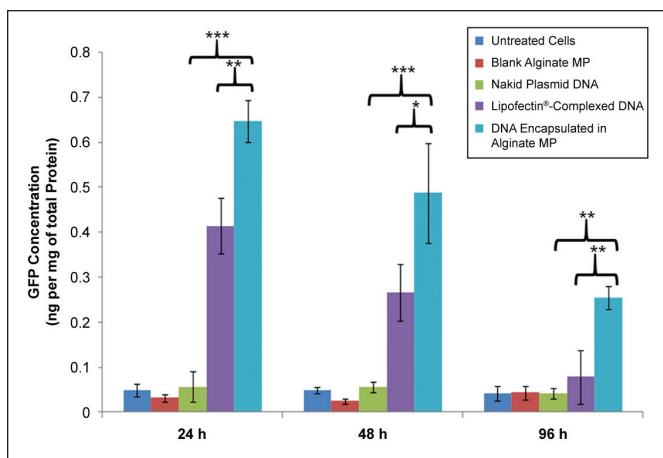
Figure 6. Quantitative evaluations of EGFP-N1 plasmid DNA transfection using green fluorescent protein (GFP)-specific ELISA in J774A-1 macrophage cells after 24 hours, 48 hours, and 96 hours post-transfection with control and DNA-encapsulated calcium alginate microparticle formulations. The plasmid DNA dose was maintained constant at 20 μg per 200,000 cells. The amount of GFP is expressed in (ng) per mg of total cell protein, which was measured using the BCA assay. The values are reported are mean $\pm$ SD (n = 3). Statistical significance of results was determined using one-way ANOVA and Tukey's Multiple Comparison Test with a 95% confidence interval ($p < 0.05$).
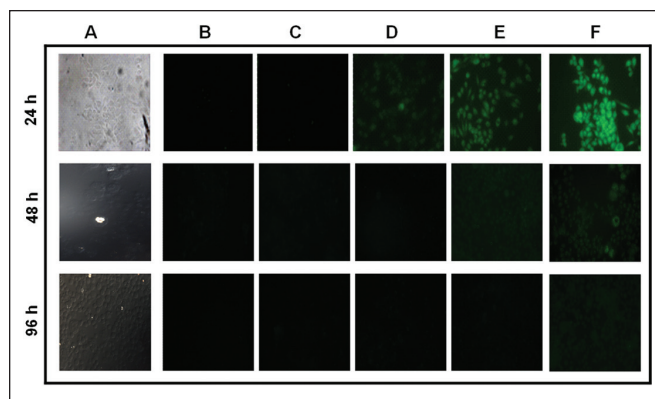

Figure 7. Qualitative evaluation of EGFP-N1 plasmid DNA transfection in J774A-1 macrophage cells after 24 hours, 48 hours, and 96 hours post-transfection with control and DNA-encapsulated calcium alginate microparticle formulations. Differential interference contrast (DIC) images of treated cells (A), and fluorescence images of untreated cells (B), and cells treated with blank microspheres (C), naked EGFP-N1 plasmid DNA (D), EGFP-N1 plasmid DNA complexed with Lipofectin® (E), and EGFP-N1 plasmid DNA encapsulated in calcium alginate microspheres. The plasmid DNA dose was maintained constant at 20 μg per 200,000 cells. All of the images were acquired at $40 \times$ original magnification.

was evident in both the Lipofectin® and alginate formulation was evident by 24 hours of particle administration. In addition, much lower fluorescence intensity also was observed in the naked plasmid treatment group. The same trend was observed at 48 hours, where the Lipofectin® and formulation treated groups again showed significant fluorescence intensity. However, at 96 hours of particle administration, the signal intensity from the alginate particles treated group was much higher as compared to Lipofectin®. The signal intensity for the naked plasmid DNA dropped significantly from 24 hours onward. These results indicated that DNA-loaded calcium-alginate particles can afford higher transgene expression for up to 4 days post-transfection.

> These results provide encouraging evidence for development of a macrophage-targeted anti-inflammatory gene delivery system with potential to treat many acute and chronic debilitating diseases.

Discussion

Gene therapy has become an exciting prospect for the treatment of the inflammatory diseases as the traditional methods lack the ability to efficiently deliver proteins and nucleic acids, especially in case of chronic inflammation where therapeutic level of the drug needs to be maintained for an extensive period of time.[15,16] However, for effective gene delivery the payload needs to be protected from the intracellular (endosomes or phago/lysosome compartment of cells) and extracellular (serum proteins/enzymes) barriers. Therefore, researchers have utilized both viral and non-viral vectors to improve the transfection efficiency of plasmid DNA.[17] However, a major drawback with viral counterparts is the associated oncogenecity and immune-genecity. Similarly, cationic condensing non-viral gene delivery vectors, such as Lipofectin® and PEI that form electrostatic complexes with the negatively charged DNA, can be highly cytotoxic to the cells or prevent release of the DNA for nuclear entry.[18,19] Therefore, the motivation behind using this system stems from the superior safety/toxicity profile and the non-condensing nature, based on physical encapsulation of plasmid DNA, of the anionic alginate matrix.

Using high viscosity grade sodium alginate, we were able to optimize formulation to reproducibly obtain particles of around 1 μm in diameter. Plasmid DNA encapsulation efficiency was optimized to be around 65% and the stability of plasmid DNA was confirmed due to processing conditions - *Figure 4*. Cell uptake of alginate particles with encapsulated rhodamine dextran was evaluated using fluorescence microscopy in J774A.1 adherent cells. Cytotoxicity analysis showed that the blank and DNA-loaded particles did not induce overt toxicity to the cells at doses that were subsequently used for DNA delivery and transfection - *Figure 5*.

DNA delivery and transfection were performed with EGFP-N1 plasmid. The quantitative GFP expression by ELISA and qualitative analysis by fluorescence microscopy showed that the alginate microparticles were most effective

as gene delivery vectors in J774A.1 macrophages - *Figures 6 and 7*. Although the exact mechanism of calcium alginate matrices in promoting phago/lysosomal escape has not been well examined, the report from You, *et al*[20] suggests that the Ca^{2+} ions used for cross-linking alginate may be sequestered by intracellular phosphate and citrate ions leading to an increase in the osmotic pressure, which will facilitate swelling and rupture of the phago/lysosomes.

Conclusions

Macrophages play an important role in acute and chronic inflammatory reactions in the body. In this study, we have investigated calcium ion crosslinked alginate microparticles as a non-condensing DNA delivery system for transfection in macrophages. Using reporter plasmid DNA expressing GFP, we have showed enhanced uptake by macrophages and the system was found to be relatively non-toxic to the cells in comparison to positive control, such as PEI. The quantitative and qualitative analysis of GFP expression was highest with calcium alginate microparticles as compared with all other controls, including Lipofectin®-complexed DNA. These results provide encouraging evidence for development of a macrophage-targeted anti-inflammatory gene delivery system with potential to treat many acute and chronic debilitating diseases.

Acknowledgements

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