Non-Condensing Calcium Alginate Microspheres for Macrophage-Selective Gene Delivery and Transfection

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ABSTRACT

The main objective of the study was to design a non-condensing polymeric microsphere-based gene delivery system for targeting macrophages. An external gelation technique was used to prepare plasmid DNA-encapsulated calcium alginate microspheres. The microspheres were characterized for particle size, surface charge, surface morphology, and DNA loading efficiency. Stability of encapsulated DNA was assessed by agarose gel electrophoresis. Microsphere uptake and transfection efficiency was examined in J774-A1 murine macrophage cell line. The results of this study show that non-condensing calcium alginate microspheres can be formulated for macrophage-specific delivery and DNA transfection.

1. INTRODUCTION

Inflammation is a defense mechanism adopted by the body in response to the variety of stimuli including pathogens, injury, and autoimmune responses (1-3). The vascularized connective tissues including plasma, circulating cells, blood vessels, cellular and extra-cellular components are capable of exhibiting an inflammatory response (1). The inflammatory response is marked by redness, heat, swelling and pain. The process is also marked by enhanced vascular permeability, recruitment of leukocytes and release of inflammatory mediators. The process of inflammation can be broadly divided into acute and chronic inflammation.

In acute inflammation, the response lasts for only few hours to few days. The main characteristics of acute inflammation are exudation of fluid and plasma proteins due to altered permeability leading to edema and the emigration of leukocytes, primarily neutrophils (1, 3). On the other hand, chronic inflammation lasts for longer duration of time and is typically a more intense response, which includes recruitment of lymphocytes and macrophages, tissue necrosis, proliferation of blood vessels, and fibrosis (1).

The primary functions of macrophages in inflammation include antigen presentation, phagocytosis, and modulation of immune response through production of various cytokines and growth factors (1,4). As *"antigen presenting cells"* (APC) , macrophages express class II major histocompatibility complex (MHC) type of molecules on the cell surface (1). After engulfing and degrading antigens early in the infectious process, the activated macrophages translocate the fragments of these antigens to cell surface where they are recognized by the T-cell receptors (TCR) on $CD4^+$ T-cells. These cells are then able to initiate cellular and humoral immunity by secretion of various cytokines. The $CD4^+$ cells are further divided into two types depending on the type of cytokine secreted. These are Th1 (T-helper-1) cells and Th2 (T-helper-2) cells. The Th1 cells secrete interleukin-2 (IL-2) and IFN-γ and antibody responses of IgG2a isotype in mice. On the other hand, Th2 cells secrete IL-4 and IL-5 and induce antibody responses of IgG1 and IgE type in mice (5,6).

Macrophages also serve as regulators of immune/inflammatory systems. As mentioned above, these cells are capable of producing an array of cytokines that can control various aspects of the immune response (1). For example, IL-12 can stimulate the helper T cells to produce an immune response and thus cell-mediated immunity. It also plays a role in regulating the movement of leukocytes from blood to tissues. On the other hand, IL-10 acts as a potent down-regulator of an inflammatory response. Thus, macrophages represent an interesting prospect for anti-inflammatory therapy perspective.

This study is aimed at employing anti-inflammatory gene therapy targeting macrophages using a safe and effective non-viral gene delivery vector. Our hypothesis for this project is that a non-condensing cross-linked calcium alginate microsphere-based DNA delivery system will be superior in macrophage transfection.

Towards this end, a non-condensing polymer, sodium alginate was considered as a delivery vehicle for our study. Sodium alginate is used in a number of food and pharmaceutical products and is considered as a *"generally regarded as safe"* (GRAS) excipients material by the U.S. Food and Drug Administration (7). Sodium alginate has been explored for its ability to retain water, gel formation in the presence of multivalent cations such as (Ca^{2+}), viscosifying, and stabilizing properties (8).

2. EXPERIMENTAL METHODS

2.1 Formulation and Characterization of Cross-linked Alginate Microspheres

The external gelation method was employed for the production of cross-linked calcium-alginate microspheres. A 3.0 ml of sodium alginate solution was filled into a 5 ml syringe fitted with a 30G-1/2-inch needle. The alginate solution was added drop-wise into calcium chloride solution (27 ml) while stirring at 2400 rpms using a 4-blade lab stirrer. The resulting suspension was centrifuged at 10,000 rpm for 35 minutes. The pellet was washed twice with deionized water. 2 ml of de-ionized water was added to the pellet and the resulting suspension was slowly freeze-dried using liquid nitrogen and the sample was then freeze-dried at -80°C, followed by lyophilization. As part of surface modification scheme, Pluronics® F108 and Poly(ethylene glycol) (PEG; Mol. wt = 10,000 Da) were incorporated into the sodium-alginate solution, at a concentration of 0.1% (w/w) of sodium-alginate, prior to cross-linking with calcium. The resulting, cross-linked microspheres were characterized for size, surface charge, and morphology by using Coulter particle sizer, Brookhaven's ZetaPALS, and scanning electron microscopy (SEM), respectively.

2.2 Determination of Plasmid DNA Loading and Stability

Plasmid DNA encapsulation in cross-linked alginate microparticles was analyzed by using the PicoGreen® dsDNA fluorescence assay. The aim of this experiment was to determine the DNA loading efficiency in the calcium-alginate microspheres. The particles were prepared in the same fashion as the blank formulation above. 20 µgs of plasmid DNA encoding for enhanced green fluorescent protein (EGFP-N1, Clontech) was first mixed with PEG. This mixture was then added to the mixture of sodium alginate and Pluronics® F108. The resulting mixture was then added drop-wise into a stirring calcium-chloride bath. The plasmid DNA loaded microspheres were then processed via the same procedure as blank particles mentioned above.

The lyophilized formulation was then evaluated for plasmid DNA loading efficiency and stability within the microspheres. Towards this end, 10 mg of lyophilized formulations were dissolved in alginate lyase solution of concentration 1mg/ml and incubated for 24 hours at 37° C. The enzyme was obtained from *Flavobacterium sp* (Sigma Chemicals, 100 mg). The suspension was centrifuged at 13,000 rpm for 30 minutes. Both the supernatant and pellet were collected for determination of plasmid DNA loading efficiency using PicoGreen® assay and gel-retardation assay for DNA stability. Before performing the gel-electrophoresis, the plasmid DNA was extracted from the degraded alginate microspheres via DNA precipitation.

2.3 Qualitative Determination of Transfection Efficiency in J774A-1 Macrophages

The aim of this study was to qualitatively determine the ability of the calcium alginate microspheres to transfect the J774A-1 macrophages. Approximately 300,000 cells were used for each well. A dose of 20 µg of EGFP-N1 plasmid DNA encapsulated in the calcium alginate microspheres, prepared by external gelation technique, was added to each well in a 6-well microplate. The untreated cells and cells treated with Lipofectin®-complexed DNA (20 µL Lipofectin® + 20 µg of plasmid DNA), naked EGFP-N1 plasmid DNA (20 µg), and blank calcium alginate microsphere formulation were used as controls. The cells in each well were incubated at 37°C in 5% CO_2 in serum-free media for 6 hours. Excess microspheres were then removed and the serum-free media in the wells was replaced with the complete cell culture media (DMEM with 10% FBS). The cells were then incubated for 24 hours at 37°C, 5% CO_2. Fluorescent microscopy was performed, using Olympus microscope equipped with BioQuant image analysis system to evaluate GFP

transfection in the control and microsphere-treated cells.

3. RESULTS AND DISCUSSION

As measured by the Coulter particle sizer, the mean diameter of the blank and plasmid DNA-loaded Ca-alginate microsphere formulation came out to be 1.28 μm and 0.95 μm, respectively. The average zeta potential (surface charge) values of the blank and DNA modified formulations was -8.6 mV and -13.5 mV, respectively. Similar negative charge on blank and DNA-containing formulation indicated that the payload was encapsulated in the microspheres rather than adsorbed to the surface. Figure 1 below shows the scanning electron micrographs of the blank and DNA-loaded microsphere formulations. The results of the SEM showed that the microspheres size was similar to that determined by Coulter sizer, they were spherical in shape, and appeared to have a smooth surface morphology.

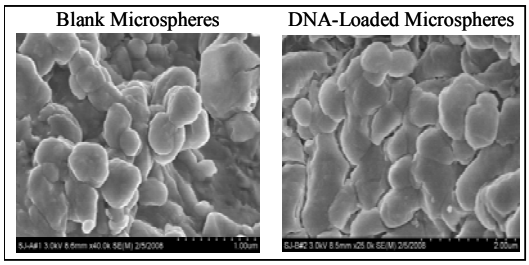

Figure 1: Scanning electron micrograph of blank calcium alginate microsphere formulation (left) and the scanning electron micrograph of plasmid EGFP-N1 DNA-loaded microsphere formulation (right).

As part of the formulation characterization study, the next step was to determine the plasmid DNA loading capacity and stability of the encapsulated DNA within the calcium-alginate microspheres. The plasmid DNA loading capacity for the microspheres was determined to be 65+/-0.1%. In order to determine the stability of the plasmid DNA within the microspheres, gel electrophoresis was performed. The results obtained after 24 hours of incubation time period with 1mg/ml and 2mg/ml alginate lyase concentration revealed bright DNA bands. The Figure 2 below shows an image of the agarose gel. DNA ladder was run on both the sides of the gel. Different bands in the DNA ladder correspond to a specific size of the DNA. Thus, the ladder acts as a marker for the tested sample. The next lane is the naked plasmid, on both the sides of the DNA ladder, precipitated by DNA precipitation method. The two bright bands in the middle represent the DNA extracted from the Ca-alginate microspheres treated with 1 mg/ml and 2 mg/ml alginate lyase (from left to right). The upper band and lower band in these lanes represent open-circular and supercoiled DNA, respectively. Also, as evident from the agarose gel image, the size of the plasmid supercoiled DNA is approximately 4.7Kb and falls between bands 8 and 9 of the DNA ladder. Most importantly, it was confirmed from the gel retardation assay that the DNA is stable within the Ca-alginate microspheres.

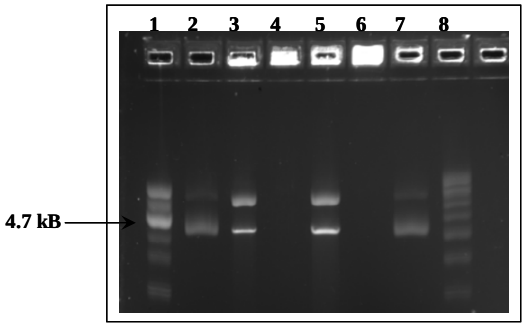

Figure 2: Stability of encapsulated DNA by agarose gel electrophoresis. Lanes 1 and 8 are 2-10 kB DNA ladder, lanes 2 and 7 are naked EGFP-N1 plasmid DNA after precipitation showing two bands corresponding to open circular and supercoiled DNA, lane 3 is EGFP-N1 plasmid DNA extracted from the microsphere formulation after incubation with 1mg/ml alginate lyase for 24 hours, lane 4 is the pellet obtained after centrifuging the formulation treated with 1mg/ml alginate lyase, lane 5 is EGFP-N1 plasmid DNA extracted from the formulation after incubation with 2mg/ml alginate lyase for 24 hour, and lane 6 is the pellet obtained after centrifuging the formulation treated with 2mg/ml alginate lyase.

Lastly, in order to qualitiatively determine the ability of the EGFP-N1 plasmid DNA loaded Ca-alginate microspheres, to transfect the macrophages, was compared with various other controls. The results of the transfection study indicated that after 24 hours of incubation, cells treated with plasmid DNA loaded formulation showed the highest transfection out of all the controls consisting of lipofectine, naked plasmid EGFP-N1 DNA, macrophages treated with blank formulation and untreated cells. Figure 3 below shows the DIC image and fluorescent images of

J774A-1 macrophages treated with controls and formulation containing plasmid DNA.

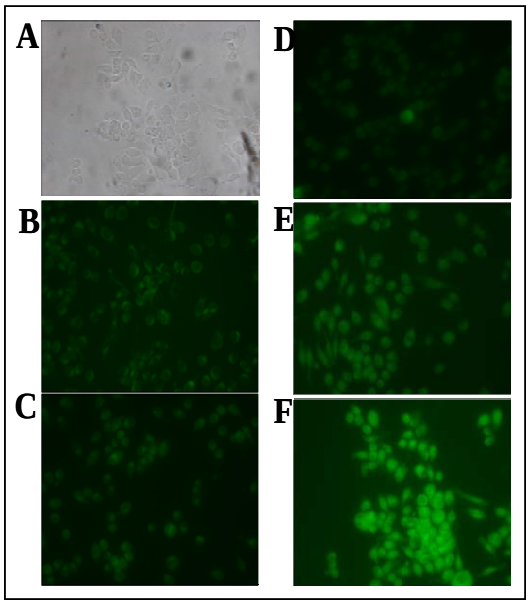

Figure 3: Qualitative evaluation of EGFP-N1 plasmid DNA transfection in J774A-1 macrophage cells. Differential interference contrast (A), and fluorescence images of untreated cells (B), cells treated with blank microspheres (C), cells treated naked EGFP-N1 plasmid DNA (D), cells treated with EGFP-N1 plasmid DNA complexed with Lipofectin® (E), and cells treated with EGFP-N1 plasmid DNA encapsulated in Ca-alginate microspheres. The plasmid DNA dose was maintained constant at 20 μg per 300,000 cells. All of the images were acquired at 40X original magnification.

4. CONCLUSIONS

The results of this study show that non-condensing crosslinked Ca-alginate microspheres have the potential to effectively encapsulate and stabilize plasmid DNA. Ca-alginate microsphere did not induce any cytotoxicity in the macrophage cell line. Additionally, the transfection studies carried out so far have shown that the Ca-alginate microspheres are far more superior then Lipofectin®, a cationic lipid-based transfection reagent, and naked plasmid DNA in terms of their ability to successfully transfect the J774A-1 murine macrophages. Future studies are aimed at determining the qualitative and quantitative tranfection efficiency of the EGFP-N1 plasmid DNA encoding micropsheres at various other time points.

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