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RESEARCH PAPER

Multi-Compartmental Nanoparticles-in-Emulsion Formulation for Macrophage-Specific Anti-Inflammatory Gene Delivery

Husain Attarwala • Mansoor Amiji

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ABSTRACT

Purpose To develop a safe and effective non-viral vector for gene delivery and transfection in macrophages for potential anti-inflammatory therapy.

Methods Solid nanoparticles-in-emulsion (NiE) multi-compartmental delivery system was designed using plasmid DNA-encapsulated type B gelatin nanoparticles suspended in the inner aqueous phase of safflower oil-containing water-in-oil-in-water (W/O/W) multiple emulsion. Control and NiE formulations were evaluated for DNA delivery and transfection efficiency in J774A.1 adherent murine macrophages.

Results Using green fluorescent protein (GFP) and murine interleukin-10 (mIL-10) expressing plasmid DNA constructs, the NiE formulation was found superior in enhancing intracellular delivery and gene transfection efficiency in cells. Anti-inflammatory effects of transfected mIL-10 were examined by suppression of tumor necrosis factor-alpha (TNFα) and interleukin 1-beta (IL-1β) production in lipopolysaccharide (LPS)-stimulated cells.

Conclusions Overall, the results were very encouraging towards development of a macrophage-specific NiE-based multi-compartmental gene delivery strategy that can potentially affect a number of acute and chronic inflammatory diseases.

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INTRODUCTION

Inflammation is a complex and highly regulated biological process, which involves sequence of events initiated as a result of extrinsic or intrinsic stimuli (1,2). Tissue-specific macrophages are one of the most important cell types of the innate immune system for executing such functions as phagocytosis, release of reactive oxygen species, expression of chemokines and cytokines, and the activation of T lymphocyte-mediated cellular immunity (1,3). Macrophages reach the inflammatory site following the initial release of chemokines and other soluble factors and migration of polymorphonuclear cells (1). Depending upon the stimuli received by macrophages, they can be differentially activated to perform variety of functions, which can trigger either a pro-inflammatory response or an anti-inflammatory response (4,5).

Macrophages activated by interferon-gamma (INF-γ), tumor necrosis factor-alpha (TNFα) and lipopolysaccharides (LPS) elicit a pro-inflammatory response by causing the release of additional pro-inflammatory cytokines and chemical mediators (4,5). On the other end, the activation of macrophages through stimulation by IL-10 and transforming growth factor-beta (TGFβ) cause down-regulation of pro-inflammatory cytokines TNFα, IL-1α and IL-1β, IL-6, and IL-12 and a decrease in the expression of major histocompatibility complex-II and co-stimulatory molecules resulting in resolution of the inflammatory response. TGFβ

promotes cellular differentiation, growth and division, and hence plays an important role in wound healing, tissue repair and regeneration (5,6). Monocytes and macrophages, when exposed to IL-10, produce soluble immune mediators, such as IL-1Ra and soluble TNFα, which additionally can bring about resolution of inflammation and help in tissue repair. Furthermore, IL-10 expression leads to inhibition in the release of many other pro-inflammatory cytokines (7,8). The pleiotropic anti-inflammatory properties of IL-10-stimulated macrophages make them a valid target for treatment of different acute and chronic inflammatory conditions.

Gene therapy involves delivery of nucleic acid constructs to specific cells of the body, where the gene encoding a therapeutic protein is expressed at sustained levels within the region of interest resulting in prevention or treatment of a disease (9). Viral vectors used for majority of gene therapy studies present significant risk of cytotoxicity, immunogenicity, mutagenesis and chromosomal integration of the delivered genes, which may trigger neo-oncogenesis. In order to overcome these serious toxicity concerns of viral vectors, there is a need for development of safe and effective non-viral gene delivery systems (10). Optimum gene delivery vehicles should not degrade the payload during transit, improve DNA bioavailability to the tissue and cell of interest, and prevent premature clearance by the reticular-endothelial systems (RES), should release the DNA in cytosol after endosomal escape upon cellular entry, should facilitate nuclear uptake of intact DNA, and result in high levels of gene transfection for sustained period of time (11,12). Although cationic lipid and polymeric carriers have been suggested, their use has been limited due to cytotoxicity, aggregation of lipo- and poly-plexes, and rapid clearance by mononuclear phagocytic system of the RES. The cationic charge on these particles facilitates their opsonization by plasma proteins like IgM, complement C, and coagulation proteins, which can enhance the recognition and rapid clearance from the body. Aggregation of lipo- and poly-plexes may also lead to deposition of emboli in lungs and small blood vessels. Overall, these systems have shown relatively poor transfection efficiencies and are prone to high toxicity concerns following systemic administration (13).

Alternatively, non-condensing hydrogel-type polymeric nanoparticles are of special interest as they can physically encapsulate plasmid DNA and avoid all the problems associated with cationic lipids and polymers (14–22). Since the DNA is encapsulated within the polymeric matrix, repulsion between DNA molecule and cell membrane, which may prevent endocytosis, is restricted. Moreover, absence of the cationic charge and surface modification with poly(ethylene glycol) (PEG), limits recognition of the non-condensing polymeric delivery vehicle by the mononuclear phagocytic system of RES, and can reduce premature clearance upon systemic administration. In addition, lack of cationic charge also avoids the possibility of activation of innate immune system causing acute inflammatory response (12,13). Non-condensing DNA encapsulating polymers like PEG, poly (ethylene oxide) (PEO), poly(D,L-lactide-co-glicolide) (PLGA), Pluronics® or poloxamer block copolymers, gelatin, and cellulose derivatives have been studied and have shown noticeable improvements in systemic gene transfection efficiency (12).

Type B gelatin-based nanoparticle vector for gene delivery and transfection, developed and optimized in our lab, has proven to be safe and highly efficient expression system upon oral and systemic administration (15,16,18). Nanoparticles formulated from type B gelatin have several advantages including biocompatibility, biodegradability, high DNA loading capacity, efficient cellular uptake by non-specific or receptor-mediated endocytosis, and high levels of transgene product formation. Type B gelatin, which is obtained by alkaline hydrolysis of collagen, has an isoelectric point between 4.8 and 5. At neutral pH, type B gelatin possesses a net negative charge, and hence can interact with DNA in a charge independent manner probably through physical encapsulation in a hydrogel matrix (16,21).

To further the development of non-viral gene delivery in macrophages for anti-inflammatory therapy, in this study, we have formulated **n**anoparticles-**i**n-**e**mulsions (NiE) multi-compartmental system. An emulsion based delivery system is attractive for several reasons. First, as a liquid formulation, it can be administered either orally or parenterally for variety of gene delivery applications. Second, the oil phase of the emulsion could also serve as another vehicle for administration of hydrophobic molecules, such as small molecular weight anti-inflammatory agent for synergistic effect. Third, the oil phase also protects the encapsulated DNA from degrading enzymes. Lastly, the oil droplet size and surface characteristics can be tailored for macrophage-specific phagocytosis. Plasmid DNA constructs encoding for green fluorescent protein (GFP) and murine IL-10 (mIL-10) were encapsulated in type B gelatin nanoparticles, which were further suspended in the inner aqueous phase of safflower oil-containing water-in-oil-in-water (W/O/W) multiple emulsion. Intracellular delivery and trafficking studies were carried out in J774.A1 adherent macrophage cells. Following observation of GFP and mIL-10 transfection efficiencies using control and NiE formulations, the suppression of pro-inflammatory cytokines, TNF-α and IL-1β, in mIL-10 transfected cells was examined at the transcriptional and translational levels in lipopolysaccharide (LPS)-stimulated cells.

MATERIALS AND METHODS

Preparation and Characterization DNA-Containing Formulations

Preparation and Characterization of Type B Gelatin Nanoparticles

Gelatin nanoparticles (GNP) were prepared by the water-ethanol solvent displacement method described previously (17). Briefly, 100 mg of type B gelatin (pI 4.5 to 5.5) having a bloom strength of 225 (Sigma-Aldrich, St Louis, MO) was dissolved in 10 mL of deionized water at 37°C. Following pH adjustment to 7.0 using 0.2 M NaOH solution, gelatin particles were precipitated out from the aqueous solution using four times as much quantity of absolute ethanol. The hydroalcoholic suspension of precipitated particles was centrifuged at 35,000 rpm for 45 min. The nanoparticle pellet was washed with deionized distilled water and freeze-dried.

Formulation of Plasmid DNA-Containing W/O/W Multiple Emulsion and Nanoparticles-in-Emulsion Formulations

The W/O/W multiple emulsion (ME) was formulated by a two step emulsification process as described by Shahiwala and Amiji (23). Extra virgin grade safflower oil, obtained from Jedwards, Inc. (Quincy, MA), was used for the external oil phase. The first step consisted of formulating W/O primary emulsion using 1.6 mL of safflower oil and 400 μL of oil-soluble surfactant Span® 80. For the preparation of ME and NiE formulations, GFP or mIL-10 expressing plasmid DNA, at a final concentration of 50 μg/mL was either directly dissolved in the 2 mL of aqueous phase or DNA-containing GNP were suspended in the aqueous phase used for preparing the primary emulsion. The aqueous and the oil phases were mixed and homogenized at 9,000 rpm for 15 min using a Silverson L4RT homogenizer to produce the primary W/O emulsion. Four-mL of the primary W/O emulsion or nanoparticle dispersion in oil was mixed with an additional 4 mL of aqueous phase consisting 20 μL of water-soluble surfactant Tween® 80 and the DNA-encapsulated formulations were prepared by homogenization at 4,000 rpm for 4 min.

Plasmid DNA Loading and Stability Studies

Determination of DNA Encapsulation Efficiency

EGFP-N1 plasmid loading efficiency in GNP, ME, and NiE formulations was evaluated by using PicoGreen® dsDNA reagent (Invitrogen, Carlsbad, CA). A standard curve was constructed with EGFP-N1 plasmid DNA complexed with PicoGreen® reagent in phosphate buffered saline (PBS, pH 7.4). For DNA loading in gelatin nanoparticles, 1 mg of lyophilized sample was incubated with 0.2 mg/mL protease in PBS for 30 min at 37°C until a clear solution was obtained. For ME and NiE formulations, the encapsulated DNA was extracted by first destabilizing the emulsion with 1/10th volume of 5 M NaCl followed by centrifugation at 20,000 rpm for 30 min and extraction of DNA from the aqueous phase (for ME) or from the extracted gelatin nanoparticles (for NiE). The extracted DNA was complexed with PicoGreen® reagent and the loading capacity and efficiency was determined using the standard curve.

Stability of Encapsulated DNA

Agarose gel electrophoresis, using 1.2% E-Gel® with SYBR Safe™ (Invitrogen, Carlsbad, CA), was performed to evaluate the stability of plasmid DNA encapsulated in GNP, ME, and NiE formulations and to ensure the integrity of the payload due to the various formulation processes. In each case, the EGFP-N1 plasmid was extracted from the formulations as discussed above and 100 ng was added to the wells for electrophoretic mobility. A 2–10 kB DNA ladder, purchased from Invitrogen, was also added to lanes 1 and 9 of the gels as size marker.

Cellular Uptake and Intracellular Trafficking Studies

Cell Culture Conditions

Adherent murine macrophages cells (J774A.1) were obtained from ATCC (Rockville, MD) and cultured in standard growth medium consisting of DMEM modified with 10% fetal bovine serum and 5% penicillin/streptomycin. Dilution medium used for the transfection studies consisted of DMEM modified with 5% penicillin/streptomycin.

Rhodamine Dextran-Encapsulated Formulations

To evaluate ME and NiE uptake in macrophages, rhodamine-labeled dextran (Mol. wt. 70 kDa) was encapsulated in the internal aqueous phase of the primary W/O emulsion or in gelatin nanoparticles prior to formulating into the final product. In the case of NiE, 40 mg of 0.5% (w/w) rhodamine-labeled dextran encapsulated gelatin particles were added to the internal aqueous phase of the multiple emulsion, while in case of ME formulation 8 μl of 25 mg/mL solution of rhodamine-labeled dextran was incorporated in the internal phase of multiple emulsion.

Cellular Uptake Studies

200,000 J774A.1 cells were plated in each well of a six-well plate containing alcohol-sterilized glass cover slip using growth medium and allowed to attach to the cover slips for 12 h prior to treatment with the control and test formulations. The cells were then fed with rhodamine-labeled dextran encapsulated in ME or NiE formulations and allowed to incubate for different time periods. Blank ME and NiE formulations were used as controls. After treatment, the J774A.1 cells were washed with sterile PBS and visualized with an Olympus fluorescence microscope.

Intracellular DNA Trafficking Studies

DNA trafficking studies in the cells are important to determine the exact pattern of intracellular release and nuclear uptake over time. EGFP-N1 plasmid DNA was labeled by chelation with PicoGreen® dsDNA reagent (Invitrogen, Carlsbad, CA). Type B gelatin was covalently labeled with rhodamine B isothiocyanate (St. Louis, MO) at 0.5% (w/w) concentration and the purified conjugate was used for nanoparticle formulation as described above. For trafficking experiments, the NiE formulation was prepared using PicoGreen®-labeled DNA molecules encapsulated in rhodamine-labeled GNP. The ME formulation was made by dissolving PicoGreen®-labeled DNA and rhodamine-labeled dextran in the inner aqueous phase. Approximately, 200,000 J774A.1 cells were plated in a six-well microplate and treated with GNP, ME, and NiE formulations in growth medium and incubated for different time points. The cell nuclei were stained with Hoechst® 33245 dye (Invitrogen, Carlsbad, CA).

After incubation of cells with the formulations for various time points, they were washed with PBS and images were acquired using a Nikon Eclipse® TE-200 fluorescence microscope having mercury arc lamp for visualizing the red (from nanoparticles), green (from DNA), and blue fluorescence (from the nuclei) at excitation/emission wavelength of 560/55 nm, 515/30 nm, and 460/50 nm, respectively.

Green Fluorescent Protein Expression Studies

Quantitative Gene Transfection Studies

J774A.1 cells were seeded in different T25 flasks and incubated at 37°C for 12 h for cell attachment. After 12 h, the growth media was replaced with the dilution media containing EGFP-N1 plasmid-encapsulated (20 μg per 200,000 cells) GNP, ME, or NiE formulations or EGFP-N1 complexed with Lipofectin®. Untreated cells and cells treated with blank formulations served as negative controls. After 6 h, the cells were washed with sterile PBS and the growth media was added again followed by incubation at 37°C for different time periods. At the completion of each time point, the cytosolic protein was obtained by cell lysis and used for analysis of GFP expression by ELISA. Additionally, the total protein concentration in the cell lysate was obtained using the Pierce's biscinchoninic acid (BCA®) protein assay (24).

GFP-specific ELISA plates were prepared in-house by coating the surface of each well of a 96-well microplate with 100 μl of mouse monoclonal anti-GFP antibody (Novus Biologics, Littleton, CO) at 1:4,000 dilution in PBS of 1 mg/mL stock. After 2 h of incubation at room temperature, wells were washed 5 times with PBS-Tween80 to remove unbound antibodies. Then non-specific binding sites were then blocked by using starling superblock (Fisher Scientific, Milwaukee, WI). The wells were washed with PBS-Tween®80 at least 5-times and then to each well, 100 μl of known GFP standards or cell lysate were added followed by incubation at 4°C overnight. After this step, all wells were washed 5 times with PBS-Tween80 and incubated with 100 μl of secondary rabbit polyclonal anti-EGFP antibody attached to alkaline phosphatase enzyme (Novus Biologics, Littleton, CO) at 1:1000 dilution in PBS of 1 mg/mL stock. After 2 h of incubation at room temperature, the wells were washed 5 times with PBS-Tween80 and incubated with alkaline phosphate substrate for 30 min. The reaction was stopped by addition of 0.5 N NaOH and then absorbance was read at 408 nm using Bio-Tek® Synergy HT microplate reader (Winoski, VT).

Qualitative Gene Transfection Studies

EGFP-N1 plasmid DNA was encapsulated in GNP at 1% (w/w), in ME at 50 μg/mL and NiE at 50 μg/mL. DNA complexed with Lipofectin®, a commercially available positively charged lipid transfection reagent, was used as a positive control. Blank formulations were used as negative controls. In each well of a six-well microplate, alcohol sterilized cover-slips were placed. Approximately 200,000 adherent alveolar macrophage cells were added in each well and allowed to adhere for 12 h at 37°C. After this period, the cells were treated for 6 h with EGFP-N1 plasmid-containing GNP, ME, and NiE formulations. A 20 μg dose of EGFP-N1 plasmid was used per 200,000 cells. After incubating the cells at 37°C with growth media for different time periods, the cover-slips were removed, washed with sterile PBS, and GFP expression was visualized using an Olympus fluorescence microscope.

Murine IL-10 Gene Transfection Studies

Isolation of mIL-10 Plasmid DNA from Transformed E. coli

Freeze-dried pellet of transformed *E. coli* producing mIL-10-expressing plasmid DNA (pORF5-mIL-10) were obtained from Invivogen (San Diego, CA). For bacterial culture, liquid agar broth was prepared by dissolving LB Agar (Sigma-Aldrich, St. Louis, MO) in deionized distilled water and sterilized using an autoclave. The bacterial suspension in LB Agar broth was then streaked on solid agar plates and incubated at 37°C for growth of colonies. After 12 h, a single colony was picked using a sterile inoculation loop and transferred to liquid agar broth culture medium containing 10 μg/mL ampicillin (for selection of mIL-10 transformed *E. coli)*. The *E. coli* were cultured for 16 h at 37°C and the plasmid DNA extraction, and purification was performed using Qiagen Plasmid Mega® kits. Purity of plasmid extracts was analyzed by measurement of 260/280 nm absorbance ratio. Quantification of DNA concentration was carried out using PicoGreen® dsDNA assay. Further confirmation of the plasmid purity and the mIL-10 cDNA size was obtained by restriction digestion with NheI and NcoI enzymes and agarose gel electrophoresis.

mIL-10 mRNA Expression Analysis by RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) is a technique for identification of gene expression at the mRNA level. For qualitative analysis of mIL-10 expression at the transcript level, 200,000 J774A.1 cells were treated with mIL-10 expressing plasmid DNA encapsulated in GNP, ME, and NiE. Additionally, naked plasmid DNA and DNA-complexed with Lipofectin® were used as controls. A plasmid DNA dose of 20 μg was administered per 200,000 cells in all of the formulations. Similar to transfection with EGFP-N1 plasmid, the media was changed after 6 h and the transfection efficiency was evaluated periodically starting from 6 h to 24 h post-administration. The total cellular RNA was extracted using High Pure® RNA isolation kit (Roche, Indianapolis, IN) and quantified using NanoDrop® 2000c UV/VIS spectrophotometer (ThermoFisher Scientific, Wilmington, DE). The isolated RNA fractions were converted to final PCR products using selective primers for IL-10 and beta-actin using Qiagen's One Step® RT-PCR kit (Valencia, CA). The forward and reverse primer sequences used for conversion of IL-10 mRNA in to cDNA were 5′-CCAGCCTTATCGGAAATGA-3′ and 5′-TCTCACCCAGGGAATTCAAA-3′, respectively. The forward and reverse primer sequences used for conversion of β-actin mRNA into cDNA were 5′-GTTACCAACTGGGACGACA-3′ and 5′-TGGCCATCTCCTGCTCGAA-3′, respectively. The final PCR products were run on 1.2% agarose E-gels (Invitrogen, CA) for visualization of cDNA bands using Carestream FX (Rochester, NY) imaging system.

mIL-10 Protein Expression Levels with ELISA

For quantitative analysis of mIL-10 protein expression in J774A.1 cells upon transfection with pORF5-mIL-10 plasmid DNA, the GNP, ME, and NiE formulations were administered such that 20 μg of the plasmid could be dosed per 200,000 cells. Naked plasmid DNA and DNA-complexed with Lipofectin® were used as controls. Since mIL-10 is a secreted protein, periodically the cell culture media was removed and the levels of mIL-10 in the media was measured using Quantikine® sandwich ELISA, purchased from R&D Systems (Minneapolis, MN).

Suppression of Inflammatory Cytokines in LPS-Stimulated Macrophages

In order to evaluate mIL-10 gene transfection mediated suppression of pro-inflammatory cytokines, TNFα and IL-1β, the J774A.1 cells were stimulated with LPS. For these studies, the cells were plated in different wells of 6-well microplates. Following cell attachment, they were treated with pORF5-mIL-10 plasmid DNA-containing GNP, ME, and NiE formulations at 20 μg DNA dose per 200,000 cells. Naked plasmid DNA and DNA-complexed with Lipofectin® were used as controls. Following 6 h of incubation with the formulations, the cells were allowed to produce mIL-10 for a period of up to 72 h. LPS at a concentration of 100 ng/mL was added to the transfected cells 6 h prior to completion of the studies. Cells that were not transfected with mIL-10 plasmid DNA, but stimulated with LPS, were used as a control.

TNFα and IL-1β Expression Analysis by RT-PCR and ELISA

After completion of each time point, the total cellular RNA was extracted from cells and converted to final PCR products

Table I Particle Size and Surface Charge (Zeta Potential) Values of Blank and EGFP-N1 Plasmid DNA-Encapsulated Type B Gelatin Nanoparticles

Nanoparticle Formulation	Hydrodynamic Diameter (nm)	Zeta Potential (mV)
Blank Gelatin Nanoparticles	164.3 ± 5.0*	−10.7 ± 0.12
DNA-Encapsulated Gelatin Nanoparticles	166.0 ± 5.6	−11.2 ± 1.35

*Mean ± S.D. (n=3)

Table II Plasmid DNA Loading Capacity and Efficiency in the Control and Nanoparticles-in-Emulsion Formulations

Formulations	DNA Loading Efficiency (%)
Gelatin Nanoparticles (GNP)	99.0 ± 0.50*
W/O/W Multiple Emulsion (ME) Formulation	54.4 ± 3.01
Nanoparticles-in-Emulsion (NiE) Formulation	70.3 ± 2.21

*Mean ± S.D. (n=3)

using selective primers for TNFα, IL-1β, and β-actin using One Step® RT-PCR kit (Qiagen, Valencia, CA). The forward and reverse primer sequences used for conversion of mRNA into cDNA for TNFα were 5′-CATGAGCACA GAAAGCATGATC-3′ and 5′-CCTTCTCCAGCTGGAA GACT-3′, respectively. The forward and reverse primer sequences used for conversion of mRNA into cDNA for IL-1β were 5′-GGCTGCTTCCAAACCTTTGA-3′ and 5′-GCTCATATGGGTCCGACAGC-3′, respectively.

For quantitative determination of TNFα and IL-1β protein expression profiles, Quantikine® ELISA kits from R&D Systems (Minneapolis, MN) were used. Cell culture supernatants obtained from each treatment group at different time periods were assayed for TNFα and IL-1β levels.

Data Analysis

The quantitative results are reported as mean ± standard deviation. Comparisons between the groups were made using student's t-test, and with more than two groups, ANOVA was used to compare the results. The values were considered statistically significant at 95% confidence interval (i.e., $p<0.05$).

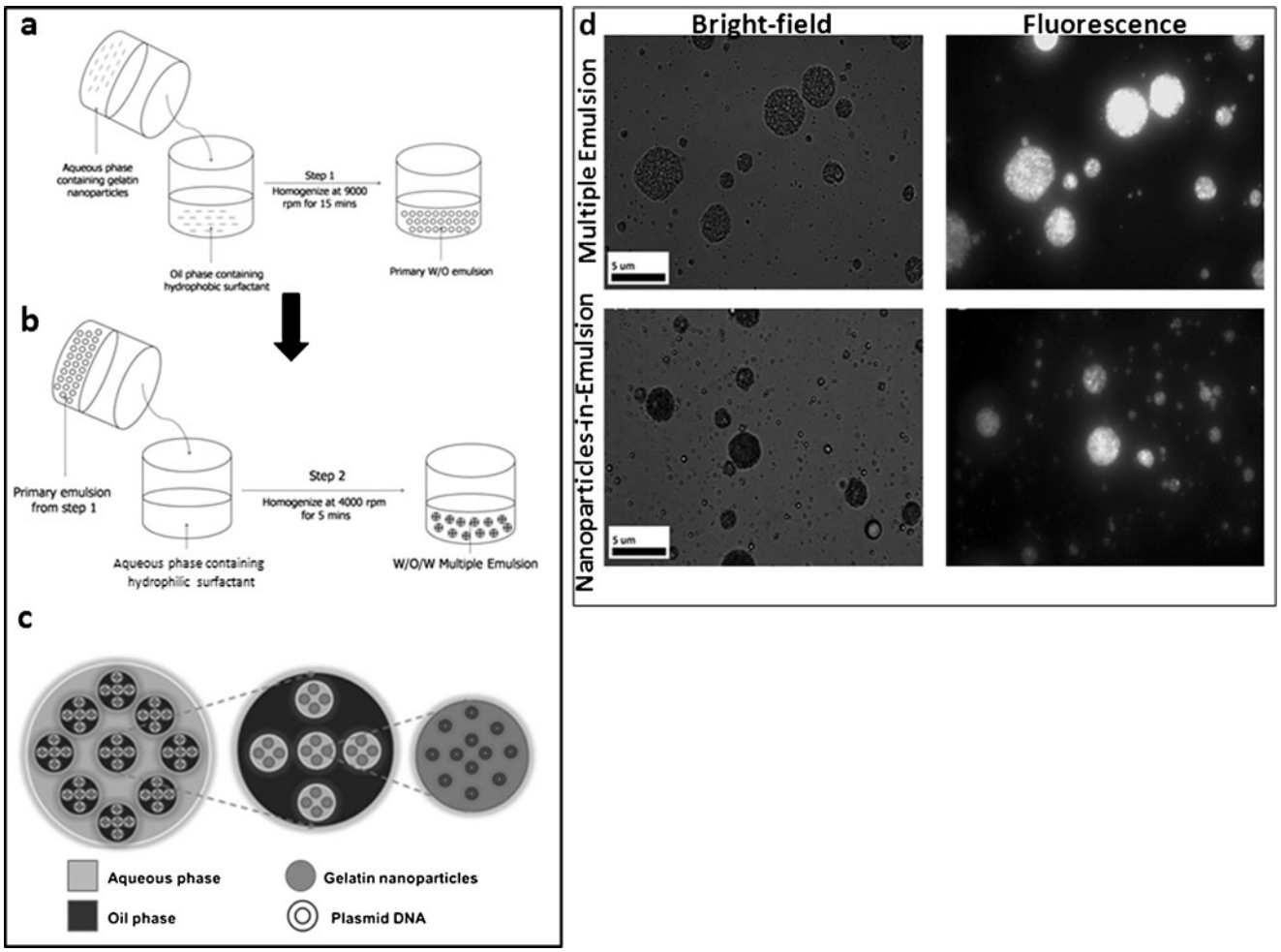
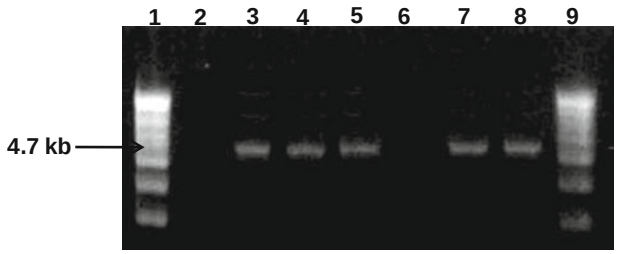


Fig. 1 Schematic illustration of the method for preparation of water-in-oil-in-water (W/O/W) multiple emulsion (**a**) and incorporation of plasmid DNA-containing type B gelatin nanoparticles to form nanoparticles-in-emulsion (NiE) multi-compartmental delivery system (**b**). The formulation has internal aqueous phase with suspended gelatin nanoparticles, the middle oil phase, and the external aqueous phase (**c**). Bright-field and fluorescence microscopy show that the oil droplets of NiE had a diameter of less than 5 μm and the rhodamine-labeled gelatin nanoparticles were completely encapsulated in the internal aqueous phase of formulation (**d**).



Lane 1: 2-10kb supercoiled DNA ladder
Lane 2: Blank NiE formulation.
Lane 3: EGFP-N1 plasmid DNA isolated from NiE formulation.
Lane 4: EGFP-N1 plasmid DNA isolated from W/O/W emulsion
Lane 5: EGFP-N1 plasmid DNA isolated from gelatin nanoparticles
Lane 6: Blank gelatin nanoparticles
Lane 7: Naked EGFP-N1 plasmid DNA as a control
Lane 8: EGFP-N1 plasmid DNA isolated from ME formulation after spiking with the plasmid.
Lane 9: 2-10kb supercoiled DNA ladder

Fig. 2 Agarose gel electrophoresis for the determination of EGFP-N1 plasmid DNA stability upon encapsulation in type B gelatin nanoparticles and further incorporation into the nanoparticles-in-emulsion (NiE) and water-in-oil-in-water (W/O/W) multiple emulsion delivery vehicles.

RESULTS AND DISCUSSION

Formulation Development and Characterization

Plasmid DNA-Encapsulated Type B Gelatin Nanoparticles

The blank and plasmid DNA encapsulated nanoparticle size and surface charge (zeta potential) values were measured using Malvern's Zeta-sizer® either before or after ultracentrifugation of the suspension. The results in Table I show that the average hydrodynamic diameter of the gelatin nanoparticles was in the range of 170 nm and zeta potential in the range of -11 mV in the DNA-encapsulated formulation. The relatively insignificant difference between the zeta potential values of the blank and DNA-encapsulated nanoparticles suggests that the plasmid DNA molecules were physically encapsulated in the hydrogel matrix rather than adsorbed to the surface (21). Additionally, Table II shows the loading efficiency of plasmid DNA in gelatin nanoparticles that was in the range of 98–99%, consistent with our earlier observations (17,19,21,25).

ME and NiE Formulations

Following preparation of blank and DNA-encapsulated gelatin nanoparticles, they were encapsulated in W/O/W multiple emulsion to formulate multi-compartmental NiE as shown in Fig. 1a–c. The W/O/W ME with plasmid DNA dissolved in the inner aqueous phase was also prepared and used as a control. The hydrodynamic diameter of the oil droplet in ME and NiE formulations was around 5 μm or less, as shown in the microscopy images in Fig. 1d. In addition, when rhodamine-labeled dextran-incorporated gelatin nanoparticles were used for preparing NiE, the fluorescence emission was confined to the inner phase and did not show any bleeding. These results suggest that the gelatin nanoparticles did not escape from the inner aqueous phase after preparation of the NiE formulation. Additionally, as shown in Table II, the final DNA encapsulation efficiencies in ME and NiE formulations were optimized to 54% and 70%, respectively. The lower encapsulation efficiency of DNA in ME may be due to partitioning of the hydrophilic payload into the outer aqueous phase during the preparation step.

Additional stability of the ME and NiE formulations was evaluated using accelerated centrifugation and evaluation of phase separation. When the ME or NiE formulation was centrifuged at up to 4,000 rpm for 1 h, there was no phase separation observed under microscopic evaluations. In addition, we also examined the effect of up to 1,000 fold dilution in aqueous media on the leaching and release of rhodamine-dextran by fluorescence microscopy either when administered directly into the W/O primary emulsion to form ME or when encapsulated in gelatin nanoparticles to form NiE. These studies showed that the fluorescence was confined to the inner aqueous phase in both of these formulations even after 1,000 fold dilution.

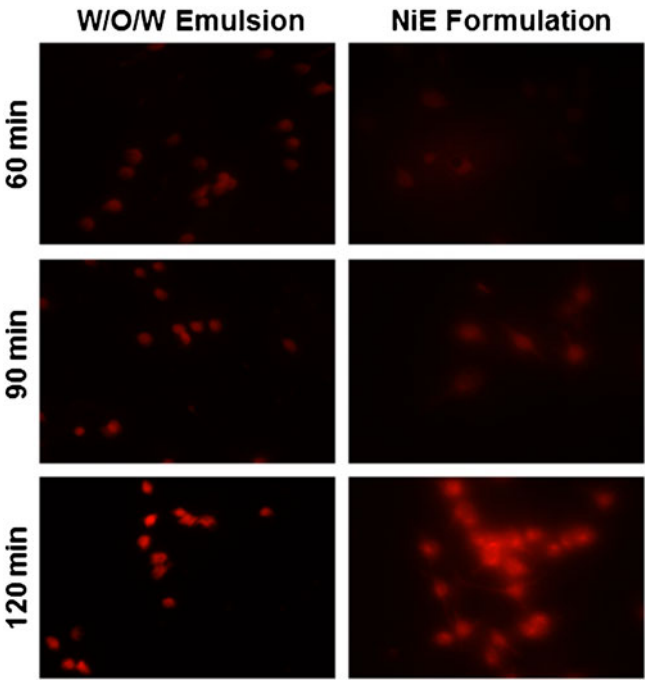


Fig. 3 Time-dependent fluorescent microscopy images of rhodamine-labeled dextran incorporated in the water-in-oil-in-water (W/O/W) multiple emulsion and nanoparticles-in-emulsion (NiE) multi-compartmental delivery system upon administration to J774A.1 adherent alveolar macrophage cells. Original magnification was 40X.

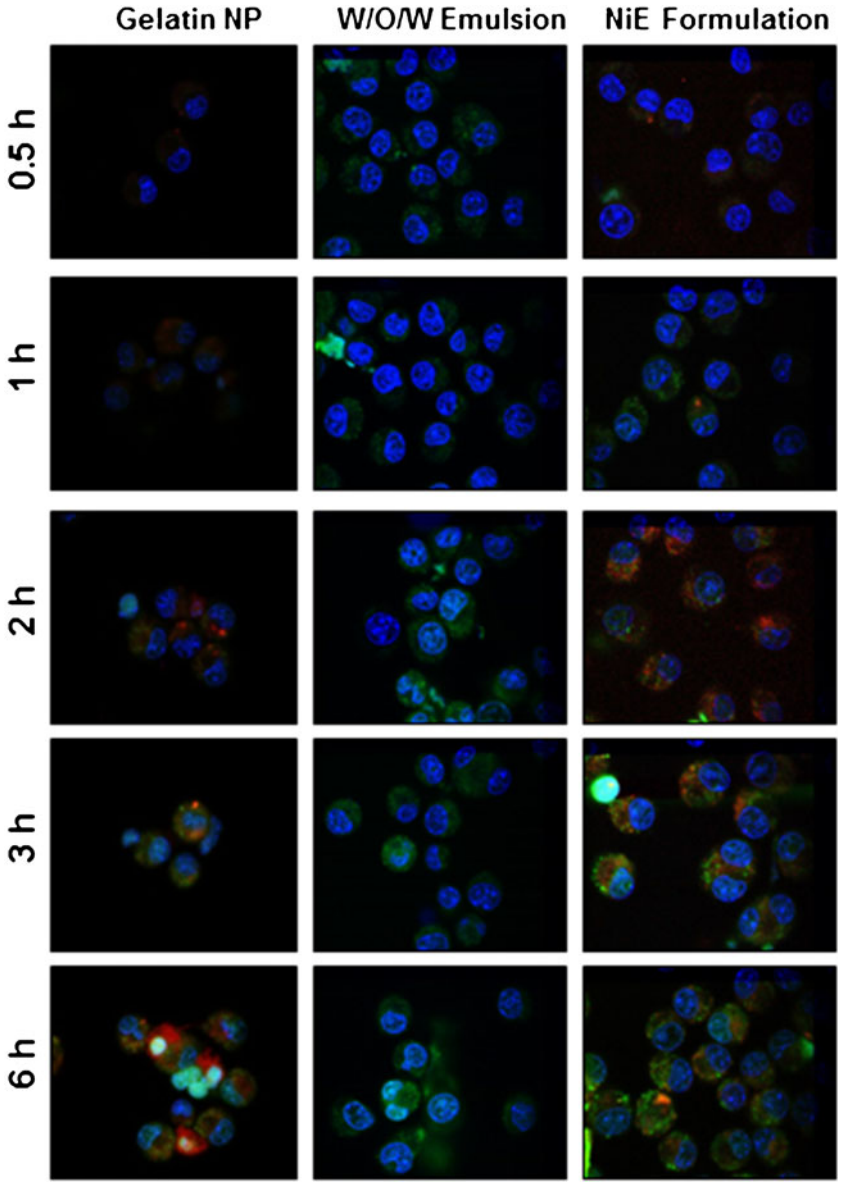


Fig. 4 Fluorescence microscopy images of EGFP-N1 plasmid DNA uptake and cellular trafficking when administered in water-in-oil-in-water (W/O/W) multiple emulsion and nanoparticles-in-emulsion (NiE) multi-compartmental delivery system to J774A.1 adherent alveolar macrophage cells. Type B gelatin nanoparticles were labeled with rhodamine (*red emission*), the plasmid DNA was labeled with PicoGreen® (*green emission*), and the nucleus of the cell was labeled with Hoechst® 33245 dye (*blue emission*). Original magnification was 60X.

Stability of Plasmid DNA in Control and NiE Formulations

The process stability of EGFP-N1 plasmid DNA in GNP, ME, and NiE formulations was evaluated by agarose gel electrophoresis. As shown in Fig. 2, the plasmid DNA extracted from the various formulations by proteolytic degradation of gelatin nanoparticles and 5 M NaCl treatment, and electrophoresed in agarose gel (lanes 3-5) yielded a single band at around 4.7 kB corresponding to the supercoiled EGFP-N1 plasmid size. These results show that plasmid DNA was not affected by the solvent displacement and shear stresses of the homogenization conditions used in the preparation of GNP, ME, and NiE formulations.

Cellular Uptake and DNA Trafficking Studies in J774A.1 Macrophages

Cellular Uptake Studies

The fluorescence microscopy images in Fig. 3 show that that the ME and NiE formulations with encapsulated rhodamine-labeled dextran were rapidly internalized by J774A.1 macrophages within the first 60 min of incubation. As time progressed, an increase in the fluorescence intensity was observed, which became maximum after 120 min of exposure. These qualitative results confirm that J774A.1 macrophages were capable of internalizing the oil droplet of ME and NiE formulation with a size of less than 5 μm.

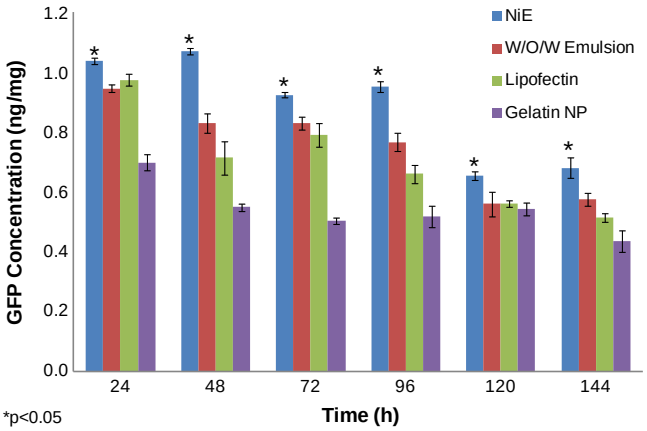


Fig. 5 Quantitative analysis of green fluorescent protein (GFP) expression by ELISA showing transgene expression with EGFP-N1 plasmid DNA encapsulated in type B gelatin nanoparticles, water-in-oil-in-water (W/O/W) multiple emulsion, and nanoparticles-in-emulsion (NiE) multi-compartmental delivery system upon administration to J774A.1 adherent alveolar macrophage cells. Intracellular GFP concentration were measured from 24 to 144 h post-administration of the formulation and reported as ng per mg of total cellular protein. Commercially-available cationic lipid transfection reagent, Lipofectin®, was used as a control.

Intracellular DNA Trafficking Studies

The trafficking studies were performed in order to assess ability of different formulations for delivery of DNA within the nuclei of the cells. Type B gelatin was covalently labeled with rhodamine and showed red fluorescence emission fluorescence, PicoGreen®-labeled plasmid DNA showed green fluorescence emission, and the cell nuclei are stained blue with Hoechst® 33245. The results, shown in Fig. 4, confirm cellular internalization of plasmid DNA-containing GNP, ME and NiE formulations starting from 30 min post-administration. At the initial 30 min time point, particles were further away from cell nuclei. Starting from 2 and 3 h time-point, the distance between the nanoparticles and nuclei was reduced and also co-localization of green and blue colors was observed with GNP, ME, and NiE formulations to suggest DNA delivery in the nucleus. Specifically in the case of NiE formulation, along with an increase in intracellular uptake of the formulations with time, there was a marked increase in nuclear co-localization of DNA with time. There was a progressive increase in particle movement from the periphery towards the cell nucleus. At 6 h time-point, for instance, the red color corresponding to the rhodamine-labeled gelatin nanoparticles was localized near the peri-nuclear space, while green color was largely co-localized with blue color to indicate nuclear entry of the plasmid DNA.

The DNA trafficking studies clearly show that NiE formulations were superior to other controls in terms of nuclear delivery of plasmid DNA. The enhanced nuclear delivery using this multi-compartmental formulation would potentially lead to higher transfection efficiency.

Reporter Gene Transfection Studies

Quantitative GFP Expression Studies

GFP-specific ELISA was used for quantitative evaluation of transfection. Following incubation of the EGFP-N1 plasmid DNA-encapsulated control and NiE formulations from 24 h to 144 h post-administration, the expressed protein in J774A.1 cells was isolated and quantified using GFP-specific ELISA. DNA complexed with Lipofectin®, a commercially-available cationic lipid transfection reagent, was used as one of the controls. The total cellular protein was quantified using BCA® assay and the results of quantitative transfection analysis were reported as ng of expressed GFP per mg of total cellular protein (Fig. 5). These results show that NiE formulation was most effective in transfection efficiency of EGFP-N1 at all of the time points tested from a single 20 µg dose per 200,000 cells. For instance, after 48 h of transfection, NiE formulation yielded 1.15 ng/mg of expressed GFP as compared to 0.81 ng/mg with ME, 0.58 ng/mg with GNP, and 0.72 ng/mg with Lipofectin®-complexed DNA. Although the concentrations of GFP decreased at longer time points, the levels were still considerably higher with NiE-induced transfection as compared to other systems tested. The GNP system, in contrast, was not as efficient in transfection probably due to rapid degradation of the matrix in protease rich endosomal/lysosomal compartments of the macrophages and subsequent rapid DNA degradation of intercellular nucleases. In the case of NiE formulation, we postulate that the encased gelatin nanoparticles were being slowly released from the oil droplets upon cellular internalization and peri-nuclear localization. Once the gelatin nanoparticles were positioned at the peri-nuclear site, the supercoiled plasmid DNA would have a short diffusional distance for efficient nuclear uptake (26,27). Additionally, the lack of cytotoxicity from the NiE formulation relative to cationic lipids (like Lipofectin®) or polymers is also another important reason for long-lasting transgene expression. As we have shown before with nanoparticles-in-microsphere oral system (NiMOS), incorporation of plasmid DNA in a non-condensing engineered gelatin-based system and protecting the nanoparticles with additional polymeric microsphere matrix affords efficient and long-lasting transfection even upon oral administration (14,15,28,29).

Qualitative GFP Expression Analysis

To further confirm the transgene expression efficiency with EGFP-N1 plasmid DNA encapsulated in control and NiE formulations, intracellular GFP was visualized by fluorescence microscopy. The images in Fig. 6 shows high levels of

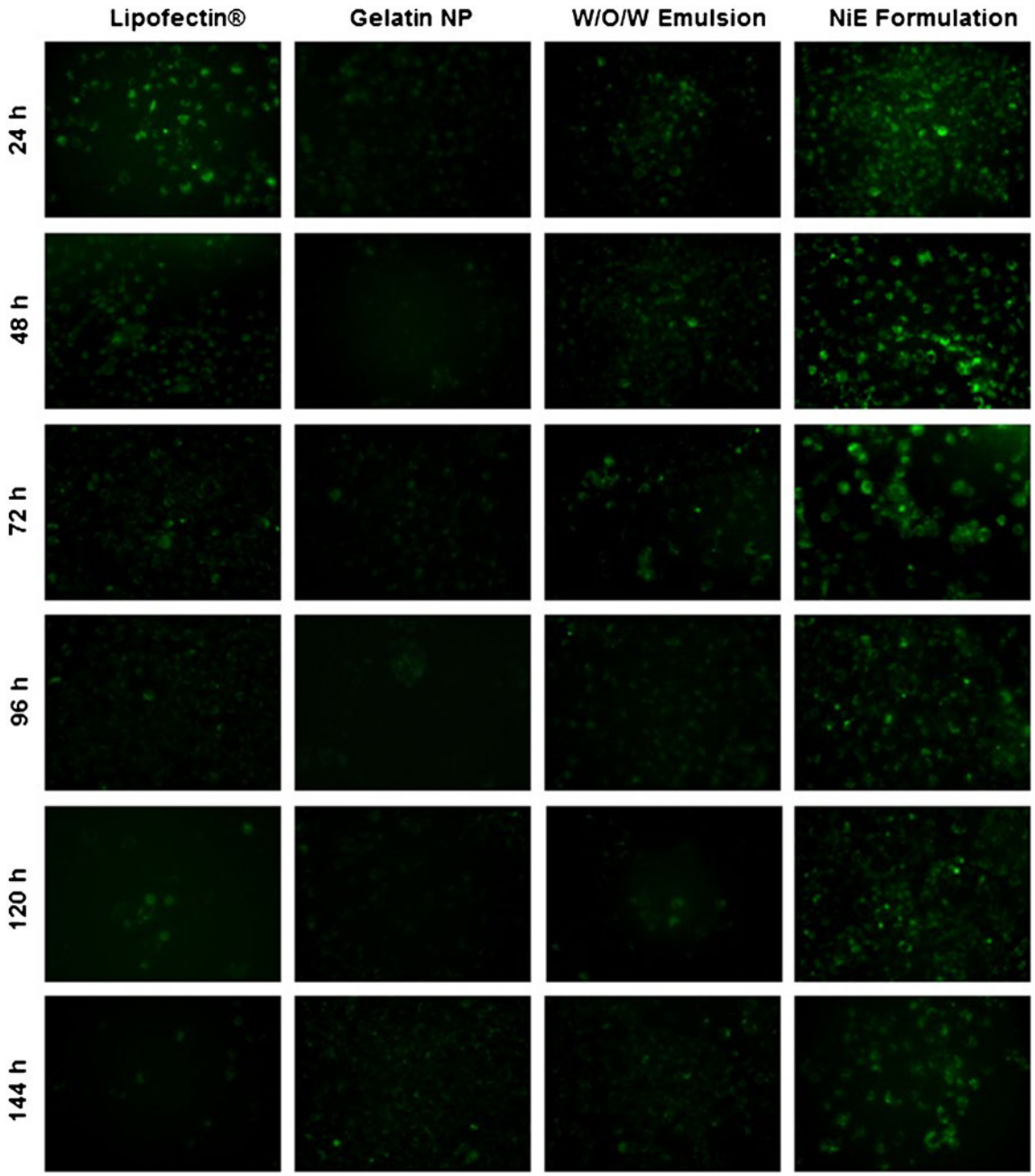


Fig. 6 Qualitative analysis of green fluorescent protein (GFP) expression by fluorescence microscopy upon administration of EGFP-N1 plasmid DNA encapsulated in type B gelatin nanoparticles, water-in-oil-in-water (W/O/W) multiple emulsion, and nanoparticles-in-emulsion (NiE) multi-compartmental delivery system in J774A.1 adherent alveolar macrophage cells. GFP expression was observed in cells treated with NiE after 24 h and remained relatively high for up to 144 h post-administration. Commercially-available cationic lipid transfection reagent, Lipofectin®, was used as a control.

GFP expression in J774A.1 cells after 24 h and up to 144 h post-transfection with DNA-encapsulated NiE formulation. Although Lipofectin®-complexed DNA showed high GFP expression at earlier time points (e.g., 24 and 48 h), the number cells decreases significantly with time due to the significant cytotoxicity of this cationic vector. In contrast, the ME or NiE formulations achieved long-lasting transgene expression due to lack of toxicity from these agents.

Murine IL-10 Therapeutic Gene Transfection Studies

RT-PCR Analysis for mIL-10 mRNA Levels

Following confirmation of transgene expression using NiE formulation with a reporter plasmid DNA, we have evaluated mIL-10 expressing plasmid DNA delivery in J774A.1 macrophages. RT-PCR analysis was carried out to compare

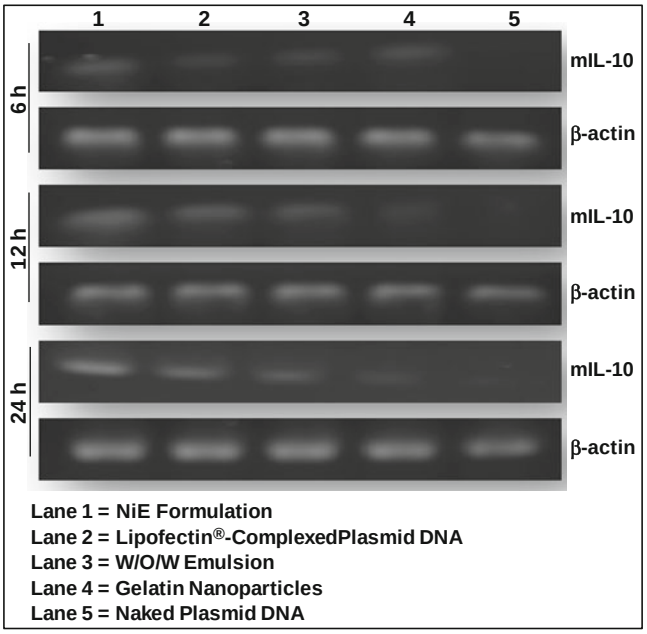


Fig. 7 RT-PCR analysis of murine interleukin-10 (mIL-10) mRNA transcript following administration of the plasmid DNA encapsulated in type B gelatin nanoparticles, water-in-oil-in-water (W/O/W) multiple emulsion, and nanoparticles-in-emulsion (NiE) multi-compartmental delivery system upon administration to J774A.1 adherent alveolar macrophage cells. Plasmid DNA-complexed with commercially-available cationic lipid transfection reagent, Lipofectin®, and administered as free (naked plasmid) were used as control. Beta-actin was used as a loading control.

the levels of mRNA transcripts in the cellular extract after 6 h, 12 h, and 24 h post-administration of the plasmid DNA-complexed with Lipofectin® or encapsulated in GNP, ME, and NiE formulations was. As shown in Fig. 7, high levels of mIL-10 mRNA transcript were observed with NiE formulation starting from 6 h post-transfection and remained stable for up to 24 h. After 12 h, the mRNA levels increased with ME, GNP, as well as Lipofectin®-complexed plasmid DNA. However, transfection with these systems caused a decrease in mRNA levels after 24 h. Administration of naked plasmid DNA did not result in any detectable mIL-10 mRNA transcript at all of the tested time points.

mIL-10 Protein Expression Analysis by ELISA

The expressed mIL-10 in transfected cells was detected in the culture medium using an ELISA assay. The results, shown in Fig. 8, represented picograms of mIL-10 per mL of the medium. Starting from 12 h post-administration of the mIL-10 expressing plasmid DNA until 96 h, there was a significant ($p<0.05$) difference in the expression of the protein using NiE formulation relative to the other reagent, including Lipofectin®-complexed DNA. For instance, after 24 h post-administration of a single 20 μg plasmid DNA dose per 200,000 J774A.1 cells, the average mIL-10 levels in the media were 200 pg/mL in cells treated with NiE formulation as compared to 110 pg/mL for ME, 75 pg/mL for GNP, and 85 pg/mL for Lipofectin®-complexed DNA. After 96 h post-administration, the mIL-10 levels were still relatively higher (160 pg/mL) for NiE-treated cells as compared to 55 pg/mL for ME-, 45 pg/mL for GNP-, and 66 pg/mL for Lipofectin®-treated cells. These results corroborate with the RT-PCR data in Fig. 7 to show that NiE was superior transfection reagent for mIL-10 plasmid DNA in J774A.1 cells as compared to all other controls.

In Vitro Anti-inflammatory Activity of Expressed mIL-10

TNFα and IL1β RT-PCR Analysis

Following confirmation of mIL-10 expression in J774A.1 cells upon administration of the plasmid DNA in control and NiE formulations, we have evaluated whether the secreted cytokine possesses anti-inflammatory effects in an *in vitro* assay. The cells were first transfected with mIL-10 expressing plasmid DNA and then stimulated with LPS 6 h before the end of the evaluation interval. The 6 h LPS stimulation at 100 ng/mL was found to cause the highest levels of TNFα and IL-1β in non-transfected cells. Using RT-PCR, the TNFα and IL-1β mRNA levels were detected after 24 h and 48 h as shown in Fig. 9. Significantly lower levels of mRNA were detected at 24 h in cells transfected with mIL-10 plasmid DNA, especially using NiE, relative to the untreated cells or those treated with naked plasmid

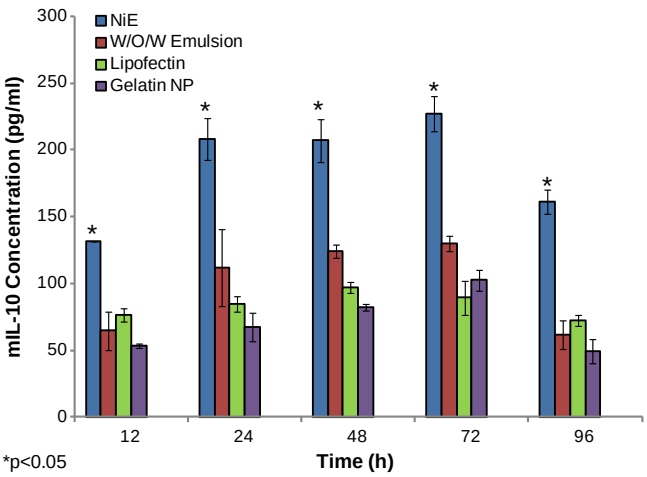


Fig. 8 Quantitative analysis of murine interleukin-10 (mIL-10) protein expression by ELISA showing time-dependent transgene expression with plasmid DNA encapsulated in type B gelatin nanoparticles, water-in-oil-in-water (W/O/W) multiple emulsion, and nanoparticles-in-emulsion (NiE) multi-compartmental delivery system upon administration to J774A.1 adherent alveolar macrophage cells. Plasmid DNA-complexed with commercially-available cationic lipid transfection reagent, Lipofectin®, was used as a control.

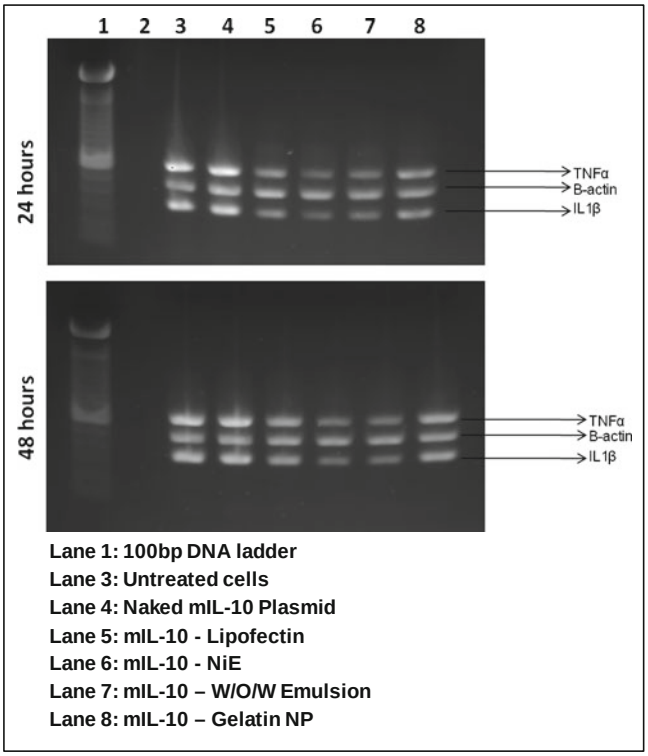


Fig. 9 RT-PCR analysis to confirm down-regulation of pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin 1-beta (IL-1β), mRNA transcript in lipopolysaccharide-treated J774A.1 adherent alveolar macrophage cells that had been transfected with murine interleukin-10 (mIL-10) plasmid DNA. The plasmid was administered in type B gelatin nanoparticles, water-in-oil-in-water (W/O/W) multiple emulsion, and nanoparticles-in-emulsion (NiE) multi-compartmental delivery system. Plasmid DNA-complexed with commercially-available cationic lipid transfection reagent, Lipofectin®, and administered as free (naked plasmid) were used as control. Beta-actin was used as a loading control.

DNA. Similarly, after 48 h post-administration of the mIL-10 expressing plasmid DNA in control and NiE formulations, there was a significantly lower TNFα and IL-1β mRNA transcript levels with NiE as compared to other formulations tested.

TNFα and IL1β Expression Analysis by ELISA

After 24 h and up to 72 h post-administration of mIL-10 plasmid DNA using control and NiE formulations in LPS-stimulated J774A.1 macrophages, an ELISA was used to quantify the concentration of TNFα and IL-1β proteins in the cell culture media. The levels of TNFα, shown in Fig. 10a, were clearly upregulated with LPS stimulation and remained relatively high even after 72 h. Upon transfection with mIL-10 expressing plasmid DNA, the lowest TNFα levels were observed with NiE formulation relative to all other systems. Plasmid DNA in ME and when complexed Lipofectin® also showed relatively lower TNFα levels as compared to GNP and naked plasmid DNA. After 48 h, for example, the TNFα levels were 2,000 pg/mL for LPS-stimulated non-transfected cells and decreased to 300 pg/mL with mIL-10 transfection in NiE formulation. The TNFα levels were 650 pg/mL in LPS-stimulated cells transfected with DNA in ME, 1,000 pg/mL with Lipofectin®, 1,100 pg/mL with GNP, and 1,700 pg/mL with naked plasmid.

Similar to the TNFα levels, IL1β levels in LPS-stimulated J774A.1 cells were also influenced by mIL-10 gene transfection as shown in Fig. 10b. Highest levels of IL-1β at around 240 pg/mL was observed in the culture media of non-transfected cells that were stimulated with LPS after 24 h. In contrast, in mIL-10 plasmid DNA transfected cells when administered in NiE formulation, the IL1β levels decreased to only 35 pg/mL after 24 h and remained around the same value for up to 72 h. Plasmid DNA administered in ME also showed relatively lower levels of IL-1β expression. In contrast, Lipofectin®-complexed mIL-10 plasmid DNA and administration of the naked plasmid showed the

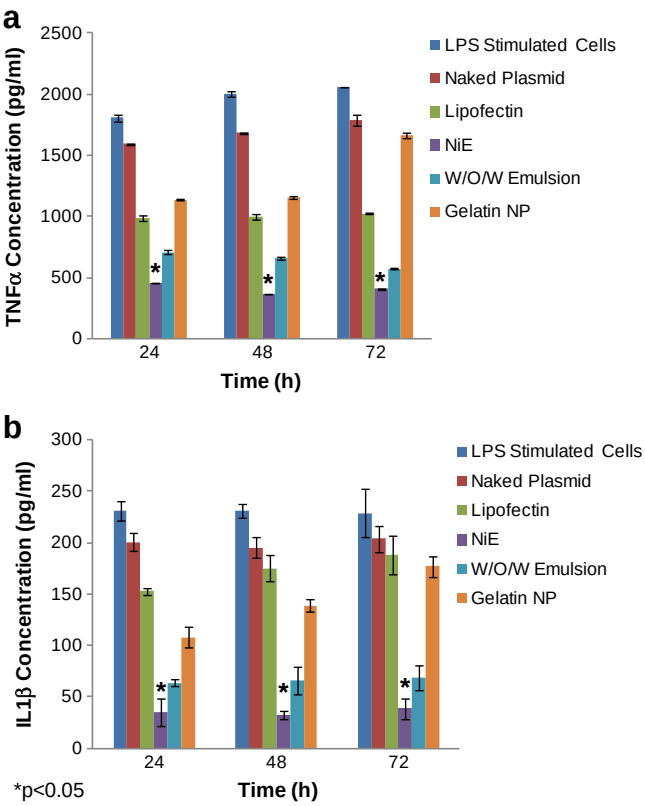


Fig. 10 Quantitative analysis to confirm down-regulation of pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) (**a**) and interleukin 1-beta (IL-1β) (**b**), proteins by ELISA in lipopolysaccharide-treated J774A.1 adherent alveolar macrophage cells that had been transfected with murine interleukin-10 (mIL-10) plasmid DNA. The plasmid was administered in type B gelatin nanoparticles, water-in-oil-in-water (W/O/W) multiple emulsion, and nanoparticles-in-emulsion (NiE) multi-compartmental delivery system. Plasmid DNA-complexed with commercially-available cationic lipid transfection reagent, Lipofectin®, and administered as free (naked plasmid) were used as control.

highest levels of IL1β expression at all of the time points, especially 48 h and 72 h.

CONCLUSIONS

The results of this study show that plasmid DNA-encapsulated solid type B gelatin nanoparticles can be further entrapped in the innermost aqueous phase of the W/O/W multiple emulsions to form stable NiE formulations. EGFP-N1 or mIL-10 expressing plasmid DNA-loaded NiE formulations were capable of producing sustained gene transfection in J774A.1 adherent murine macrophage cell line. Moreover, it was found that EGFP-N1 or mIL-10 plasmid DNA encapsulated in NiE had superior gene transfection efficiency as compared to the GNP and ME formulations, as well as, when electrostatically complexed with Lipofectin®, a cationic-lipid based transfection reagent. It was also confirmed that mIL-10 delivered through NiE was capable of exerting anti-inflammatory effect by down-regulating the expression of pro-inflammatory cytokines, TNFα and IL-1β, in LPS-stimulated cells. Overall, these results are very encouraging for potential evaluation of the NiE formulation in systemic or local gene therapy by enhancing macrophage-specific uptake.

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