In Vitro Evaluations of Macrophage-Targeted Anti-Inflammatory Gene Delivery and Transfection using Nanoparticles-in-Emulsion Formulations

Masters of Science Thesis

By

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To

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SUMMARY

The main objective of this thesis project was to develop a safe and effective non-viral vector for gene delivery and transfection in macrophages. Macrophages function as major phagocytic cells and also as antigen presenting cells. In addition to these functions, macrophages play an important role in modulation of the inflammatory response by release of different chemical mediators. Amongst the various cytokines and chemokines released by macrophages, interleukin-10 (IL-10) plays an important role in the resolution of inflammatory response. Priming of macrophages with IL-10 can bring about change in activation state of macrophages which can lead to inhibition of release of pro-inflammatory cytokines. IL-10 also exerts an anti-inflammatory effect on other immune cells like mast cells, granulocytes and T cells and hence overall brings about resolution of inflammation, tissue repair and wound healing. Hence, IL-10 can serve as a significant therapeutic agent for treatment of inflammatory diseases such as rheumatoid arthritis and intestinal bowel disease. For successful transfection of the genes in target cells, development of a non-viral vector, which is safe, efficient, and capable of producing high levels of gene expression products for a prolonged period of time, is essential. In addition, for mucosal gene delivery, the delivery vehicle should be such that it can provide sufficient protection to the encapsulated DNA from physiological fluids and enzymes at the mucosal surface until the payload is released at the desired target site for gene expression. In order to develop such a delivery system, gelatin nanoparticles were prepared and incorporated in the internal phase of safflower oil-containing water-oil-in-water (W/O/W) multiple emulsion formulations.

The project started with preparation and optimization of nanoparticles in emulsion formulation followed by plasmid DNA loading and stability studies within the formulation. Later, the formulation was tested for intracellular uptake and trafficking followed by gene transfection studies. For this purpose murine adherent alveolar macrophage cell line J774A.1 was used. Cellular uptake studies were carried out using fluorescence microscopy with J774A.1 cells
treated with rhodamine-labeled dextran encapsulated formulations. Qualitative and quantitative
gene transfection studies were carried out using a reporter plasmid EGFP-N1 encoding green
fluorescent protein and therapeutic plasmid mIL-10 encoding murine IL-10 cytokine. For
qualitative gene transfection studies using reporter plasmid fluorescence microscopy was used,
while for quantitative evaluation of gene transfection, GFP specific ELISA was used. Followed
by these studies, nano particles in emulsion formulation was tested for therapeutic mIL-10 gene
transfection using reverse transcriptase polymerase chain reaction (RTPCR) and mIL-10
specific ELISA analysis. Gene transfection studies showed high levels for transgene expression
in cells treated with the nanoparticles-in-emulsion formulation as compared to other groups of
control formulations. Furthermore, expression IL-10 in transfected J774A.1 macrophages cells
showed reduced expression of pro-inflammatory TNFα and IL-1β cytokines upon
lipopolysaccharide stimulation.

Overall, the results of the study were very encouraging towards development of
macrophage-specific anti-inflammatory gene therapy strategy that can potentially affect a
number of diseases. Additional in vivo studies will be required to fully understand the potential of
this system.
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1. INTRODUCTION

1.1 Role of Macrophages in Inflammation

1.1.1 The Inflammatory Response: Inflammation is a highly complex and highly regulated biological process, which involves sequence of events initiated as a result of extrinsic or intrinsic stimuli [1, 2]. The cardinal signs of inflammation include redness, heat, swelling and pain. Inflammation plays a significant role in protecting the body from injury and from invasion by pathogens like bacteria and viruses [2, 3]. Depending upon the duration of inflammatory response it is categorized as either acute inflammation or chronic inflammation where acute inflammation lasts for a shorter duration ranging from few minutes to several days, while chronic inflammation lasts for a much longer duration [1]. Acute inflammation is characterized by increase in vascular permeability, exudation and edema followed infiltration of leukocytes at the local site of inflammation [2]. In case of chronic inflammation, lymphocytes and macrophages appear at the site of inflammation along with angiogenesis, fibrosis and tissue necrosis at the sites of inflammation [1]. The inflammatory response must be tightly regulated because either deficiency or excess of inflammatory response can lead to various diseased conditions [4].

1.1.2 Functions of Macrophages: Macrophages present one of the most important cell types of the innate immune system for executing different functions like phagocytosis of the invading microorganisms and also the foreign particles or debris left behind after cell destruction. Another important role of macrophages is expression of different proteins or enzymes, release of reactive oxygen species, chemokines and pro-inflammatory or anti-inflammatory cytokines (Table 1). A third function of macrophages is presentation of antigen to T lymphocytes for activation of cellular immunity [1, 5]. The process of inflammation is initiated with the release of chemokines and soluble factors by vascular endothelial cells, dendritic cells,
fibroblasts and interstitial cells. These lead to enhanced vascular permeability, edema, recruitment and extravasations of innate immune cells. Polymorphonuclear cells (PMNs) are the first to migrate to the inflammatory site, followed by macrophages and lymphocytes. Macrophages bring about mobilization, activation and regulation of effector cells such as leukocytes and lymphocytes which are involved in inflammatory/immune response. [1]. Hence macrophages play an important role in induction and modulation of inflammatory response.

Table 1. Inflammatory mediators secreted by macrophages (Adapted from ref. [6])

| LTBA, LTC4, LTD4, 5-hydroxyicosatetraenoic acid and thromboxanes |
|---|---|
| Platelet-activating factor |
| Platelet-derived growth factor (PDGF) |
| PGE2, PGF2 and PGD2 |
| Histamine |
| Macrophage-derived mucous secretagogues (MMS-68) |
| Proinflammatory cytokines: | IL-1, TNF-α, IL-6, IL-8 |
| Chemokines: | MIP1α, RANTES, MIP1β, MCP-1, MCP-3 |
| Immunoregulatory cytokines: | IL-10, IL-12, IL-16 (lymphocyte chemoattractant factor), GM-CSF |
| Nitric oxide (NO), endothelins, superoxide anions, β-glucuronidase and neutral proteases |

GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; LTBA, leukotriene B4; LTC4, leukotriene C4; LTD4, leukotriene D4; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGF2, prostaglandin F2; RANTES, regulated on activation normal T cell expressed and secreted; TNF-α, tumor necrosis factor α.

1.1.3 Activation of Macrophages: Macrophages can be differentially activated to perform different functions [7]. Different patterns of macrophage stimulation can lead to activation of
either a pro-inflammatory, anti-inflammatory or Th2 dependent immune pathway (Figure 1) [7, 8]. These different patterns of macrophage activation are reviewed below.

1.1.3.1 Classical Macrophage Activation: Classically activated macrophages are the macrophages which are activated by exposure to two signals. The first signal is given by INF-γ which primes the macrophages for activation. The second signal is given by TNF either exogenous or endogenous. Physiological endogenous production of TNF is brought about by agonists of toll like receptors such as Lipopolysaccharides (LPS).

**Figure 1:** Differential expression of functional phenotype of macrophages under the influence of specific immune mediators (reproduced from ref. [9]).

Hence for macrophages to be classically activated signals from INF-γ and TNF are essential [7]. These macrophages are also referred to as M1 activated macrophages [8]. Once macrophages are activated by the classical pathway they migrate to the inflammatory site and destroy causative pathogens. These activated macrophages have enhanced capability of killing or degrading intracellular microbes due to release of NO and toxic oxygen species like O₂ [7].
1.1.3.2 Alternatively Activated Macrophages: Macrophages activated by alternate pathway are also called M2 cells. Martinez et al. classified alternatively activated macrophages in to three sub categories namely M2a, M2b and M2c depending upon their pathway of activation and the function performed. M2a subtypes of macrophages are those activated by exposure to IL-4 or IL-12 produced mainly by mast cells, basophils and TH2 cells. These cells express high levels of fibronectin 1 and betaIGH3 (a matrix associated protein) which help in promoting fibrogenesis. M2a cells promote tissue repair and regeneration by expression of coagulation factor XIII and insulin like growth factor 1. These cells also affect the IL-1β activity by promoting production of IL-1R antagonist. Activation of IL-1β is prevented by down regulation of caspase 1 which brings about proteolytic cleavage of pro-IL-1β into its active form.

In addition to these, M2a cells do not posses cell killing activity due to absence of iNOS and presence of high levels of arginase 1, which deviates the metabolic pathway of NO production to synthesis of proline and polyamine which have a role in collagen formation, tissue repair and cell growth (Figure 1). Hence M2a cells take part in resolution of inflammation, tissue repair and wound healing [7, 8]. Activated macrophages termed as M2b cells are activated through Toll like receptor stimulation (LPS) and IL-1 in presence of ligation of immune complexes to Fc receptors of IgG. These cells differ from M1 cells in terms of production of high levels of IL-10 and low levels of IL-12 thereby eliciting a type II immune response. These cells do not entirely elicit an anti-inflammatory response as these cells in addition to IL-10 also release TNF, IL-6 and IL-1β. Another subtype of macrophages termed as M2c cells are deactivated through stimulation by IL-10 and TGFβ. These are called deactivated macrophages since they bring about down regulation of pro-inflammatory cytokines - TNFα, IL-6 and IL-12 through IL-10 stimulation. Stimulation through IL-10 in addition to decreasing levels of pro-inflammatory cytokines also decrease antigen presentation efficiency of macrophages and monocytes by downregulation of MHC II complex and other co stimulatory molecules on macrophage surface. TGFβ elicits its anti-inflammatory effect by modulating cytokine
production, chemotaxis and macrophage activation by LPS. Also, TGFβ promotes cellular differentiation, growth and division and hence play a role in wound healing, tissue regeneration and repair [8, 10]. Hence, sequential activation of macrophages by different signals leads to expression of first a proinflammatory phenotype which helps in elimination of the causative pathogen, followed by expression of an anti-inflammatory phenotype which helps in resolution of inflammation preventing undesired damage to normal body cells and tissues.

Release of cytokines by macrophages can modulate their own functions. After activation of macrophages by LPS, chemokines are released in the first 8 hours. In next 16 hours, IL-8 is released which is because of TNFα and IL-1 production. Furthermore, release of TNFα leads to release of IL-10 which inhibits release of TNFα, IL-1α/β, IL-6, IL-8 and G-CSF which bring about resolution of inflammation [11].

1.2 Role of IL-10 in Inflammation

In 1989, Mosmann and his co workers described a cytokine produced by type 2 T helper cells capable of inhibiting INF-γ production by type 1 T helper cells. They termed this cytokine as cytokine synthesis inhibitory factor (CSIF) which is now called Interleukin 10 [12]. IL-10 is produced by T cells, B cells, antigen presenting cells, mast cells and granulocytes amongst which macrophages are the major producers of IL-10 [13, 14]. IL-10 plays a significant role in immune regulation by its action on different cell types (Table 2).

Monocytes and macrophages when exposed to IL-10 bring about synthesis and release of soluble immune mediators which bring about resolution of inflammation and help in tissue repair. These mediators include IL-1RA and soluble TNFα receptor. In addition IL-10 brings about inhibition in release of anti-inflammatory cytokines including IL-1β, IL-6, G-CSF, GM-CSF and TNFα. IL-10 also reduces the antigen presenting ability of monocytes and macrophages by down-regulating MHC-II surface molecules, co-stimulatory molecules like CD86 and adhesion molecules such as CD58. IL-10 also inhibits production of IL-12 by
monocytes which plays a key role in cell mediated immunity. IL-10 enhances expression of IgG-Fc receptors and scavenger receptors on monocytes and macrophages, which brings about an increase in their phagocytic activity which is important for clearance of apoptotic cells and debris resulting from the inflammatory response. IL-10 promotes suppression of IL-12 production by T

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<th>Cell Population</th>
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Table 2. Effect of IL-10 on different immune cell types (Adapted from ref. [13]).
cells and other accessory cells which in turn suppress IFN-γ production by T cells and natural killer cells which brings about development of type 2 cytokine response [14, 15]. IL-10 has inhibitory effect on CD4+ T cells where proliferation of CD4+ T cells and release of proinflammatory cytokines (IL-2, IL-4 and IL-5) and IFNγ is inhibited. Also, in presence of IL-10 activation of CD4 cells results in development of regulatory phenotype of these cells. However on CD8+ T cells, IL-10 has no inhibitory effect. On neutrophils, IL-10 has inhibitory effect on chemokine release, synthesis of cyclooxygenase 2 and synthesis of prostaglandin E2. On eosinophills and mast cells, IL-10 inhibits LPS induced release of proinflammatory mediators. In IL-10 knockout out mice a fatal inflammation of intestine was observed which could be reversed by IL-10 administration which confirms role of IL-10 in suppression of inflammation. Also in many inflammatory and autoimmune animal disease models IL-10 has shown its effects in suppressing inflammation [13].

1.3 Anti-Inflammatory Gene Therapy Strategies

Gene therapy involves delivery of genes to specific cells of the body where the gene encoding a protein is expressed resulting in high concentrations of desired protein resulting in treatment or prevention of a disease. Initially gene therapy was restricted only to genetic diseases where an abnormal or missing gene was delivered to replenish function of the missing gene. However, gene therapy can also be used for treatment of non-genetic diseases by supplementing genes encoding proteins capable of modulating cellular function [16]. Different approaches for enabling delivery of genes into cells followed by expression of therapeutic protein are delivery of genes through direct injection in to target tissue [17], gene delivery through viral vectors and gene delivery through non-viral vectors [18]. With advances in molecular biotechnology, our knowledge about immune mediators promoting or inhibiting inflammation has increased. This has facilitated identification of molecules which can modulate inflammatory response and can be used as therapeutic agents for treatment of inflammation.
Amongst regulators of inflammatory response, cytokines play an important role in regulation of entire inflammatory process. Various cytokine targeted protein and gene therapies have been explored in recent past. Protein therapies, such as soluble TNF-receptor (etanercept), anti-TNF antibody (infliximab), IL-1 receptor antagonist (IL-1Ra), were found to be efficacious [19] but these required repeated high dose injections because of very short protein half life. Another limitation of protein therapy is high systemic protein concentrations after injection compared to protein concentration at target site of action [16]. This may lead to undesired side effects of the therapeutic protein molecule at sites other than the target site in the body. These limitations can be overcome if genes encoding therapeutic protein are expressed in target cells leading to levels of protein which are high at target tissue compared to levels of protein in systemic circulation. This leads to reduced systemic side effects. Also, in case of gene therapy repeated administration of therapeutic genes is not required as one copy of plasmid DNA delivered inside a cell can undergo multiple expression cycles over a prolonged period of time, hence requiring reduced frequency of dosing and also reduced systemic side effects [16]. It was demonstrated in different studies concerning anti-inflammatory gene therapy where expression of cytokines such as IL-4, IL-10, IL-4IgG1 chimeric protein and Epstein barr virus-encoded vIL-10 were found to be effective in treatment of diseases such as rheumatoid arthritis, multiple sclerosis and autoimmune thyroiditis in different animal disease models [20]. Antagonists of proinflammatory cytokines such as soluble TNF receptor molecule, IFN-γR-IgG fusion molecules and IL-12 receptor antagonist have been found useful in normalizing inflammatory response mediated by their respective mediators. Most of these studies involved use of viral vectors for gene transfection. Although viral vectors have high gene transfection efficiency, their clinical use has been limited because of cytotoxicity, immunogenicity, mutagenesis and chromosomal integration of delivered genes leading to oncogene activation. Hence to overcome these problems non-viral gene delivery systems were developed [21].
1.4 Non-Viral Gene Delivery Systems

Novel gene delivery vehicles capable of inducing high levels of gene transfection have been developed. Gene delivery vehicles should be such that they do not allow degradation of DNA in plasma, do not allow their clearance by reticular endothelial systems should release the payload in cytosol by endosomal escape, should facilitate nuclear entry of DNA and bring about high levels of gene transfection for a prolonged period of time. [22, 23] Lipids and polymers are generally used for non-viral gene delivery. Polymers for non-viral gene delivery can be classified into condensing polymers, which can complex DNA by electrostatic charge condensation [23, 24] and non-condensing polymers, which can physically encapsulate the DNA [23, 25].

1.4.1 Gene Delivery Using Condensing Polymers: DNA condensing polymers are cationic polymers which bring about electrostatic complexation of negatively charged phosphate groups of DNA with positively charged amine groups of polymeric material. This leads to formation of a particulate complex called polyplex which can be endocytosed and expressed by the target cells. Amongst various cationic polymers, poly(L-lysine) and poly(ethylenimine) (PEI) are the most widely studied biomaterials for gene transfer. Due to synthetic and polymeric nature of these materials, modifications like attachment of ligands and alterations in molecular weights are possible. Since the charge at cell membrane is negative, endocytosis of a positively charged particulate complex is favored because of charge interaction. This helps in increase in transfection efficiency of these systems [23, 26]. Although lipid carriers have shown high transfection efficiency, their clinical use has been limited because of drawbacks like cytotoxicity, aggregation of polyplexes and rapid clearance by mononuclear phagocyte system. Aggregated cationic polymers are readily taken up by the mononuclear phagocyte system and cleared from blood circulation. Hence, this results in reduction of transfection efficiency. Aggregation can also cause deposition of emboli in lungs and blood vessels leading to toxic side effects. Cationic charge on these polymeric particles also facilitates their opsonization by plasma proteins like IgM, complement C and coagulation proteins which bring about enhanced recognition and rapid
clearance of cationic carriers by mononuclear phagocyte system. This immune recognition of positively charged carriers could even lead to activation of innate immune system, triggering an acute inflammatory response. Such an acute inflammatory response may be desirable for DNA vaccination, which could enhance immunogenicity of delivered DNA vaccine, but in case of their use for gene therapy in inflammatory diseases, this could actually aggravate the existing diseased condition [27]. Researchers have tried to address some of the problems by chemical modification of cationic polymers. In case of poly(L-lysine), aggregation of positively charged particles can be reduced by increasing aqueous solubility of poly(L-lysine). This can be achieved by attachment of dextran to epsilon amino groups of poly(L-lysine). While in case of PEI, aqueous solubility can be increased by covalent coupling of PEG with amino groups of PEI. Also it was found that modification of poly(L-lysine) with PEG can reduce its cytotoxic effect on cells in culture [26].

1.4.2 Gene Delivery Using Non-Condensing Polymers: Non-condensing polymers are capable of encapsulating DNA by non-electrostatic interaction. These polymers possess either a neutral charge or a slight negative charge, which helps in protecting DNA from exogenous nucleases. Absence of positive charge prevents electrostatic interactions between polymer and negatively charged DNA, making DNA encapsulation more feasible. Also, since the DNA is encapsulated within polymeric matrix, repulsion between DNA molecule and cell membrane which may prevent endocytosis is restricted. Moreover, absence of positive charge limits recognition of non-condensing polymeric delivery vehicle by the mononuclear phagocyte system and hence limits early clearance of the delivery vehicle. In addition, non-cationic charge on polymer also avoids possibility of activation of innate immune system by mononuclear phagocyte system causing acute inflammatory response [23, 27]. DNA encapsulating polymers like PEG, poly(ethylene oxide) (PEO), poly(D,L-lactide-co-glicolide) (PLGA), poloxamer block copolymers, gelatin and cellulose derivatives have been studied previously and have shown noticeable gene transfection efficiency in various studies. PEG and PEO bring about DNA
encapsulation by means of hydrogen bonds between nucleotide bases and phosphate groups of DNA and molecules of polymeric matrix. Poloxamers (Pluronics®) which are PEO-based copolymers have also been used for DNA encapsulation [23]. Lemieux, et al reported a poloxamer based vector for gene transfection in skeletal muscles composed of pluronic L16 and F127 which had better transfection efficiency compared to that observed in polyvinyl pyrrolidone (PVP) based vector. Also a DNA dose 500 times smaller than that used for poly(N-vinylpyrrolidone) vector was found to be sufficient for optimal gene transfection using the pluronic polymer blend [28]. Type B gelatin-based nanoparticle vector for gene transfection was originally developed in Professor Amiji’s laboratory and has shown efficient systemic and local delivery, intracellular trafficking, and expression in various local and systemic studies [25, 29, 30]. Nano particles formulated from Type B gelatin have several advantages like biocompatibility, biodegradability, high DNA loading capacity, high gene transfection efficiency, high cellular uptake by non specific or receptor mediated endocytosis. Type B gelatin, which is obtained by alkaline hydrolysis, has an isoelectric point between 4.8 and 5. At neutral pH Type B gelatin has net negative charge on its surface hence type B gelatin and DNA interact in a charge independent manner and DNA gets physically entrapped in hydrogel like matrix of gelatin biopolymer [25, 31]. These properties of gelatin make it a polymer of choice for its use as an efficient and safe non-viral gene transfection vector.

1.5 Rationale for Nanoparticles-in-Emulsion (NiE) Formulations

One of the biggest challenges facing drug delivery industry today is delivery of peptides, proteins and nucleic acid based biological molecules through oral route. Oral administration of a drug has advantages such as ease of administration, ease of dosage regulation and dosing frequency and reduction in overall cost of therapy. For successful oral delivery of bio-molecules like nucleic acids, delivery vehicles should be designed such that they can provide protection to the encapsulated payload from physiological environment of GI tract until they release the
payload at the absorbing mucosal membrane of GI tract for cellular uptake of bio-molecule to show its therapeutic action. For gene therapy of local and systemic diseases, GI tract is an exciting target because successful gene transfection in GI tract can not only bring about production of encoded protein at the local site of transfection, but also could bring about release of synthesized protein in systemic circulation. Also the GI tract is an exciting target for delivery of DNA vaccines, where encoded protein antigen can bring about development of local and systemic immune responses [23, 32, 33]. Although successful controlled release of small molecules like therapeutic proteins and peptide antigens through water in oil in water multiple emulsion in the intestine has been reported by several research groups [32, 34, 35], there has been only few studies confirming efficient gene delivery and transfection by utilization of multiple emulsion system as a gene delivery vehicle. Although proven successful for protein delivery, in case of gene delivery, enhanced protection for DNA is essential because DNA needs to be protected not only from intestinal nucleases, but also has to reach up to nucleus to bring about effective gene transfection. To address this issue, DNA can be provided with additional protection by encapsulation within nano particles, which can be then encapsulated in the innermost aqueous phase of the multiple emulsion. In this manner a multi-compartmental DNA delivery vehicle can be formulated which can provide enhanced protection and controlled release property to encapsulated gene. Multiple emulsion globules containing plasmid encapsulated nano-particles can release its payload either before its uptake by M cells of Peyer’s patches in small intestine or after their engulfment by phagocytic cells present within payer’s patches. Since multiple emulsion globules are hydrophobic, they can readily get internalized by intestinal macrophages and other cells. Once internalized by macrophages, these cells can travel and deliver the protein in different parts of the body through systemic circulation. Also DNA encapsulated in nanoparticles can result in higher loading of DNA in the emulsion system as DNA inside nanoparticles can remain protected and hence can withstand high shear homogenization steps during process of preparation of multiple emulsion. Based on
results obtained from gene transfection studies using gelatin nano-particles in our lab, we hypothesized that if these nano particles can be provided with further protection by their encapsulation in the innermost phase of water-in-oil-in-water (W/O/W) multiple emulsion system, we could obtain controlled and enhanced gene delivery and transfection both \textit{in vitro} and \textit{in vivo}. With this hypothesis under consideration we began our project with formulation optimization followed by \textit{in vitro} evaluations of gene transfection in murine adherent alveolar macrophage cell lines J774A.1.
2. OBJECTIVES AND SPECIFIC AIMS

2.1 Statement of the Problem

Gene therapy involves treatment of disease by delivery of genes into desired target cells followed by expression of delivered gene to produce therapeutic proteins. Development of safe and efficient vectors for gene therapy has been a major concern limiting use of vast knowledge of genes that we now have after completion of human genome project. Although viral vectors have shown high gene transfection efficiencies, their clinical use has been limited because of limitations like cytotoxicity and chromosomal integration of delivered gene and oncogenicity. Hence there is a vital need for development of non-toxic and safe non-viral vectors for efficient gene delivery and transfection. Although oral route of drug administration is most favorable because advantages like ease of needle-less drug administration and reduced cost of therapy, delivery of genes through this route is a major challenge because of physiological environment around the mucosal surface acts as a major barrier for mucosal gene delivery. Based on their vital role during the inflammatory process, non-viral gene delivery systems targeted towards macrophages residing in the Peyer’s patches can provide great outcomes for treatment of different acute and chronic inflammatory diseases. Although macrophages appear as an exciting target for anti-inflammatory gene therapy, accomplishment of efficient gene transfection in these cells is a major challenge because following phagocytosis, macrophages can bring about destruction of any foreign material they encounter.

2.2 Objectives and Experimental Hypotheses

The main objective of this thesis project is to develop an a non-viral gene delivery system consisting of gelatin nanoparticles encapsulated in multiple emulsions system which can bring about efficient gene delivery. Here we hypothesize that gelatin, non-condensing polymer when encapsulated innermost phase of multiple emulsion, can bring about efficient gene transfection. Evaluations of green fluorescent protein expressing plasmid DNA (EGFP-N1)
transfection efficiency were carried out murine adherent alveolar macrophages J77A.1. Further studies will include evaluations of IL-10 gene transfection murine adherent alveolar macrophages J774A.1.

2.3 Specific Aims

AIM 1: Formulation, optimization and characterization of W/O/W multiple emulsion containing plasmid DNA encapsulated gelatin nanoparticles.

a) Formulation and characterization of stable W/O/W multiple emulsion using safflower oil.
b) Preparation and characterization of type B gelatin nanoparticles.
c) Formulation of a multi-compartmental delivery vehicle consisting of gelatin nanoparticles encapsulated in inner aqueous phase of W/O/W multiple emulsion.

AIM 2: Cellular uptake and transfection studies with reporter plasmid in macrophages.

a) Establishment and maintenance of J774A.1 macrophage cells in culture
b) Formulation of nanoparticles-in-emulsion (NiE) delivery system containing rhodamine-labeled dextran (MW 70,000) encapsulated in gelatin nanoparticles.
c) Cellular uptake and distribution of rhodamine dextran containing formulations in adherent alveolar macrophages.

AIM 3: Qualitative and quantitative evaluation of transfection efficiency of nanoparticles-in-emulsions formulations using reporter and therapeutic plasmid DNA.

a) Preparation of nanoparticles in emulsions formulations containing plasmid EGFP-N1 and plasmid DNA encoding IL-10.
b) Evaluation of qualitative transfection of plasmid EGFP-N1 and IL-10 in J774A.1 macrophages using fluorescence microscopy, RT-PCR, and western blot analyses.
c) Evaluation of quantitative transfection of plasmid EGFP-N1 and IL-10 in J774A.1 macrophages using ELISA.
3. MATERIALS AND METHODS

3.1 Preparation and Characterization Nanoparticles-in-Emulsion (NiE) Formulations

3.1.1 Preparation and Characterization of Gelatin Nanoparticles: Gelatin nano-particles (GNP) were prepared by a similar solvent displacement method optimized previously in our lab [36]. Briefly, 100 mg of type B gelatin bloom strength 225 obtained from Sigma Aldrich was dissolved in 10 ml of water at 37°C. After this step pH was adjusted to 7 using 0.2 M NaOH solution. Gelatin particles were precipitated from the aqueous solution using ethanol as an anti-solvent. The final ratio of ethanol water was optimized to 4:1. Prior to its addition to gelatin solution, the temperature of ethanol solution was brought down to -40°C in order to enhance the anti-solvent effect. Enhanced green fluorescent protein (EGFP) expressing plasmid DNA (EGFP-N1) was added at concentration of 1% w/w of solubilized gelatin. Alternatively for cell uptake studies rhodamine-conjugated dextran (Mol. wt. 70kDa) was added at final concentration of 0.5% w/w of gelatin to gelatin solution after pH adjustment for its physical encapsulation. The precipitated particles were centrifuged at 35,000 rpm for 45 minutes using a Beckman ultracentrifuge. The nanoparticle pellet was collected, washed with deionized water, flash frozen in liquid nitrogen, and lyophilized for 24 hours to give a dry powder.

3.1.2 Formulation of Nanoparticles-in-Emulsion (NiE) Formulation: Water-in-oil-in-water (W/O/W) multiple emulsions are triphasic systems composed of three phases, inner aqueous droplets contained in oil globules surrounded by outer aqueous phase. The W/O/W multiple emulsion was formulated by a two step emulsification method similar to a method described before by Shahiwala and Amiji [32]. Extra pure safflower oil (Jedwards, Inc., Quincy, MA), which is rich in omega-3 and omega-6 polyunsaturated fatty acids, was used for the oil phase of the emulsion. Safflower oil contains high levels of tocopherols [37, 38], which are reported to have a negative effect on levels of pro-inflammatory cytokines tumor necrosis factor alpha and
interleukin 1 beta[39, 40]. Also, the tocopherols have shown to exert antioxidant activity and may provide protection against acute inflammation mediated by reactive oxygen species [41].

The first step consisted of formulation of W/O primary emulsion using an oil soluble surfactant Span® 80. Naked plasmid (ME) or plasmid encapsulated in the gelatin nanoparticles (NiE) was incorporated in the aqueous phase of the primary emulsion to give the final concentration of 50 µg/ml of the W/O/W multiple emulsion. The appropriate amounts of each ingredient in the optimized formulations are listed in Table 3.

A stable primary W/O emulsion with encapsulated naked plasmid DNA or as dispersion with DNA-encapsulated nanoparticles was formed using the Silverson homogenizer L4RT at the speed of 9,000 rpm for 15 minutes. The primary emulsion of dispersion was mixed with additional aqueous phase consisting of a water soluble surfactant Tween® 80 and the formulation was prepared by homogenization at 4,000 rpm for 4 minutes. Increasing the speed to greater than 4,000 rpm in this step resulted in rupture of the multiple emulsion globules.

Table 3: The Composition of the Optimized Primary and Multiple Emulsion or Dispersion Formulations

<table>
<thead>
<tr>
<th>Composition of W/O/W</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 1 – Primary Emulsion</strong></td>
<td></td>
</tr>
<tr>
<td>Safflower Oil</td>
<td>1.6ml</td>
</tr>
<tr>
<td>Span 80</td>
<td>20%</td>
</tr>
<tr>
<td>Water</td>
<td>2ml</td>
</tr>
<tr>
<td><strong>Step 2 – Multiple Emulsion</strong></td>
<td></td>
</tr>
<tr>
<td>W/O emulsion from Step 1</td>
<td>4ml</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.25%</td>
</tr>
<tr>
<td>Water</td>
<td>4ml</td>
</tr>
</tbody>
</table>
3.2 Plasmid DNA Encapsulation and Stability Studies.

3.2.1 Plasmid DNA Loading Studies: Loading efficiency of pEGFP-N1 in NiE, ME and GNP formulations was evaluated by using Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen, Carlsbad, CA). PicoGreen® dsDNA Reagent is a highly sensitive fluorescent stain for quantification of double stranded DNA. For this purpose NiE and ME were loaded with pEGFP-N1 at final concentration of 50 µg/ml in the internal phase of multiple emulsion. GNPs were loaded with 1%w/w of pEGFP-N1 40 mg of which were incorporated in the internal phase of the multiple emulsion. Extraction of encapsulated pEGFP-N1 from lyophilized GNP was carried out by incubating 1 mg of gelatin particles with 1 ml of PBS containing 0.2 mg/ml protease for 30 minutes at 37°C. For ME formulation, pEGFP-N1 was extracted by first destabilizing emulsion by addition of 1/10th volume of 5M NaCl followed by centrifugation at 20,000 rpm for 30 minutes. After this process oil and water layers became separated into two distinct layers. After discarding oily layer, aqueous layer was diluted 200 times to concentration of 2 µg/ml with PBS. Ten µl of this diluted solution was used for estimation of DNA loading. In case of NiE 1 ml of emulsion was de-stabilized with 100 µl of 5M NaCl and centrifuged at 20,000 rpm for 30 minutes. The GNP palette obtained was treated with 5ml of protease buffer for 30 minutes at 37°C for release of EGFP-N1 plasmid DNA from the particles. Blank gelatin particles and emulsions with blank inner aqueous phase or blank gelatin particles in inner aqueous phase were used as control after same extraction treatment as plasmid loaded formulations was performed. To obtain calibration curve different known standard concentrations of EGFP-N1 plasmid DNA in PBS were prepared. To different wells of a 96 well plate 100 µl of PicoGreen® assay reagent was added followed by addition of 100 µl of either standard, sample or control solution. The 96 well plate was incubated for 5 minutes protected from light at room temperature and then fluorescence intensity was measured at 480 nm excitation wavelength and 520
emission wavelength using Bio-Tek (Winooski, VT) Synergy® HT microplate reader with KC4 software.

3.2.2 Stability of EGFP-N1 Plasmid DNA: Gel retardation assay was performed to evaluate stability of plasmid DNA encapsulated in NiE, ME and GNP to ensure physical and chemical stability of plasmid DNA during the process of formulation. For this purpose E-Gel® 1.2% with SYBR Safe™ (Invitrogen, Carlsbad, CA) were used. SYBR safe DNA stain has an advantage that it is safer compared to ethidium bromide which can cause mutations and chromosomal transformations. Agarose gel electrophoresis involves movement of DNA through agarose gel under influence of electric field in which negatively charged DNA moves towards positive end through the gel. In this process larger sized DNA fragments move slower compared to smaller sized DNA fragments through the agarose gel. Hence size dependent separation of DNA occurs over time as it travels through the gel. Bands formed after the process of electrophoresis can be visualized under blue light UV trans-luminescence. In our study, during the process of emulsification if the plasmid DNA had broken into fragments we would observe multiple bands on agarose gel. Alternatively if the plasmid DNA was stable in its supercoiled form during formulation process, we would observe a single band of plasmid DNA. To determine the size of Plasmid DNA a supercoiled DNA ladder (Invitrogen, Carlsbad, CA) was used. Three formulations containing pEGFP-N1 under our study – NiE, ME and GNP were tested for plasmid EGFP-N1 stability. EGFP-N1 plasmid DNA was extracted from NiE, ME and GNP formulations as mentioned above and loaded at concentration of 100ng in 20ul of loading buffer per well of 1.2% E-Gel cassette® (Invitrogen, Carlsbad, CA).

3.3. Cellular Uptake and Intracellular Trafficking Studies

3.3.1 Cell Culture Conditions: Adherent murine alveolar macrophages J774A.1 cells, obtained from American Type Culture collection (ATTC, Rockville, MD), were cultured in T75 culture flasks using Dulbecco’s Modified Eagle Medium (DMEM) modified with fetal bovine
serum and penicillin/streptomycin antibiotics. Cells were allowed to divide until they reached desired density. Cell count was estimated by placing 20 µl of the cell suspension mixture on a hemocytometer slide. Cell viability studies were performed using Trypan blue dye exclusion assay.

3.3.2 Cellular Uptake and Localization Studies: To evaluate NiE and ME uptake in macrophages and intracellular localization, rhodamine-dextran (Mol. wt. 70kDa) was encapsulated in the internal aqueous phase of the primary W/O emulsion or in the gelatin nanoparticles prior to formulating into the multiple emulsions. The final concentration of rhodamine-dextran incorporated in both the formulations was 0.0025% (w/v). In case of NiE 40 mg of 0.5% (w/w) rhodamine-dextran encapsulated gelatin particles were added to internal phase of multiple emulsion, while in case of ME formulation 8 µl of 25 mg/ml solution of Rhodamine dextran was incorporated to the internal phase of multiple emulsion.

Approximately 200,000 cells were plated in each well of a six-well plate containing alcohol-sterilized glass cover slip and allowed to adhere for 12 hours prior to treatment with control and test formulations. The cells were fed with multiple emulsions containing 0.0025% (w/v) rhodamine-dextran either solubilized in the inner aqueous phase or encapsulated in gelatin particles which were then added to the internal aqueous phase of W/O/W emulsion. Multiple emulsions prepared with blank aqueous phase or with blank gelatin nanoparticles were used as controls. After 60 minutes, 90 minutes, and 120 minutes following incubation with the control and test formulations, the cells were washed with sterile phosphate buffered saline (PBS, pH 7.4) and the cover slip was removed from the wells and mounted on a clean glass slide. The samples were observed with an Olympus fluorescence microscope equipped with a BioQuant image analysis system.

3.3.3 Intracellular DNA Trafficking Studies: In order to understand cellular uptake and intracellular transport of plasmid EGFP-N1 containing NiE formulation, DNA trafficking studies
were performed. Towards this end, NiE was labeled using a fluorescent dye Rhodamine B isothiocyanate (St. Louis, MO) which was covalently conjugated to GNP at concentration of 0.5% w/w, resulting in final dye concentration of 0.0025%w/v in NiE. DNA was labeled using PicoGreen® reagent (Invitrogen, Carlsbad, CA). The nuclei of the cells were stained using Hoechst® 33245 dye (Invitrogen, Carlsbad, CA). Approximately 200,000 J774A.1 macrophage cells were plated in a six well plate and treated with fluorescently labeled NiE formulation. After incubation of cells with NiE formulation for 1 hour, 2 hours, 4 hours and 6 hours time points, images were acquired using a Nikon Eclipse® TE 200 microscope having mercury arc lamp for visualizing blue, red and green fluorescence at excitation/emission wavelength of 460/50 nm, 515/30 nm and 560/55 nm. Based on different fluorescent signals emitted by NiE, plasmid EGFP-N1 and nuclei of cells, localization of NiE and plasmid EGFP-N1 relative to nuclei of cells was visualized. Similar studies were performed using rhodamine conjugated GNP alone and ME containing rhodamine-labeled dextran (Mol. wt. 70,000) dissolved in the innermost aqueous phase of the multiple emulsion.

3.4 Reporter GFP Gene Transfection Studies

3.4.1 Qualitative Gene Transfection Studies: To determine whether the above formulations were capable of causing gene transfection in murine alveolar macrophage cell lines, gene transfection studies were carried out. For this purpose, plasmid EGFP-N1, a reporter plasmid which expressed enhanced green fluorescent protein (EGFP) was used. Gene transfection studies were carried out for NiE, ME and GNP formulations. EGFP-N1 plasmid DNA was incorporated at final concentration of 50 µg/ml in to NiE and ME. In case of NiE 40mgs of gelatin particles loaded with 1% (w/w) of EGFP-N1 plasmid was added to the inner aqueous phase of the W/O/W multiple emulsion while in case of ME 400 µl of 1 mg/ml solution of EGFP-N1 was incorporated in the innermost aqueous phase of the multiple emulsion.
Transfection of EGFP-N1 plasmid DNA with Lipofectin®, a commercially available positively charged lipid transfection reagent was used as positive control. While cells treated with blank formulations were used as negative controls. In each well of a six well plate alcohol sterilized cover slips were placed. Approximately 200,000 adherent alveolar macrophage cells were inoculated in each well of a six well plate using DMEM modified with 10% FBS and 5% penicillin streptomycin and allowed to adhere to cover slip over a period of 12 hours. After this period serum containing medium was replaced with formulation containing serum free medium followed by incubation at 37°C for 6 hours in order to allow sufficient uptake of formulation by the cells. After 6 hours formulation containing medium was replaced with DMEM modified with 10% FBS and 5% penicillin-streptomycin antibiotic. The samples were observed with an Olympus fluorescence microscope equipped with a BioQuant image analysis system at day 1 day through day 6 post treatment.

3.4.2 Quantitative Gene Transfection Studies: To further confirm the results obtained by above experiment, we decided to perform quantitative gene transfection studies. For this purpose, 300,000 adherent alveolar macrophage cells were inoculated in T25 flasks. Twelve hours post inoculation cells were treated with either NiE, ME, or GNP EGFP-N1 plasmid DNA-containing formulations mentioned above. One group of cells was treated with positively charged lipid Lipofectin® complexed with EGFP-N1, which served as a positive control. Additionally, cells treated with blank formulations served as negative controls. After the cells were treated with different formulations for six hours in serum free DMEM, they were incubated for a period of 24, 48, 72, 96, 120 and 144 hours at 37°C. After completion of each time point total cytosolic proteins were extracted by cell lysis using 400 µl of cell lysis buffer at 4°C. Samples were stored at -80°C until they were used for quantification of protein levels. BCA® (Biscinchoninic acid) protein assay by Thermo Fisher Scientific was used for estimation of total protein concentration. BCA assay involves reaction of amino acid residues with Cu²⁺ ions which in turn leads to formation of Cu¹⁺ ions by a Biuret reaction. Cu¹⁺ ions formed in above reaction
reacts with biscinchoninic acid resulting in formation of a soluble purple colored complex which exhibits linear absorbance at 562 nm [42]. A calibration curve using different concentrations of albumin standard was plotted and used for estimation of total protein concentration from each sample. After estimation of total protein concentration green fluorescent protein specific ELISA was performed. ELISA plate for sandwich ELISA was prepared. Towards this end, primary anti-EGFP monoclonal antibody (Novus Biologics, Littleton, CO) at 1:4,000 dilution was added to each well for attachment of the antibody to the bottom surface of each well. After 2 hours of incubation at room temperature, wells were washed 4 to 5 times with PBS-Tween80 to remove unbound antibodies. Then non-specific binding sites were then blocked by using starling superblok (Fisher Scientific, Milwaukee, WI). Again, the wells were washed with PBS-Tween®80 approximately four or five times. Then to each well 100 µl of known standard concentrations of GFP protein or 100 µl of cell supernatant obtained after cell lysis were added followed by overnight incubation at 4°C. After this step all wells were washed 4-5 times with PBS-Tween80 in order to remove any unbound protein and then secondary rabbit polyclonal anti-EGFP antibody attached to alkaline phosphatase enzyme (Novus Biologics, Littleton, CO) was added in each well. After two hours of incubation at room temperature wells were again washed 4-5 times with PBS-Tween80 in order to remove any unbound secondary antibody molecules. After this final washing step all wells were incubated with alkaline phosphate substrate for 30 minutes. Reaction was stopped by addition of 0.5 N NaOH and then absorbance was read at 408nm using Bio-Tek® Synergy HT plate reader with KC4 software. Concentration of EGFP in samples was calculated from the standard calibration curve. Transfection efficiency was calculated as nanograms of EGFP present per milligram of total protein.
3.5 Therapeutic IL-10 Gene Transfection Studies

3.5.1 Amplification, Purification and Isolation of mIL-10 Plasmid DNA from Transformed *E. coli*: mIL-10 plasmid transformed *E. coli* bacteria were obtained from Invivogen (San Diego, CA). Liquid agar broth was prepared by dissolving 35 g of LB Agar powder obtained from Sigma Aldrich (St. Louis, MO) in 1 L deionized water and sterilized by autoclaving at 121°C for 15 minutes. Lyophilized *E. coli* bacteria disc was suspended in liquid broth agar medium. The bacterial suspension was then streaked on solid agar plates prepared as per suppliers instructions [43] and incubated for 12 hours at 37°C for growth of bacterial colonies. After 12 hours, 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 transformed *E. coli*. Culture flasks containing *E. coli* were incubated at 37°C for 16 hours on a temperature regulated incubator shaker. Following this step, plasmid DNA extraction, purification and isolation was performed using Qiagen Plasmid Mega kits as per step by step procedure detailed in the kit protocol supplied by kit manufacturer [44]. Purity of plasmid DNA extracts was analyzed by measurement of A260/A280 values. Quantification of DNA concentration was carried out using PicoGreen® assay (Invitrogen, Carlsbad, CA). For determination of size of extracted plasmid mIL-10, agarose gel electrophoresis was performed either with undigested plasmid or plasmid digested with Nhel and Ncol restriction enzymes obtained from New England Biolabs (Ipswich, MA).

3.5.2 RT-PCR Analysis for mIL-10 mRNA Expression: Reverse transcriptase polymerase chain reaction is a technique for identification of gene expression at mRNA level. For qualitative analysis of IL-10 expression at the level of mRNA, 200,000 J774A.1 cells were incubated with NiE, ME, GNP, Lipofectin® or naked IL-10 plasmid for 6, 12 and 24 hours. After completion of each time point, total cellular RNA was extracted from cells using High Pure RNA Isolation Kit (Roche, Indianapolis, IN) as per the kit protocol [45]. Quantification of isolated RNA
was performed using NanoDrop® 2000c instrument (Thermo-Fisher Scientific, Wilmington, DE). The isolated RNA fractions were converted to final PCR products using selective primers for IL-10 and beta-actin using One Step RT-PCR kit (Qiagen, Valencia, CA) as per kit manufacturer protocol [46]. The forward and reverse primer sequences used for conversion of IL-10 mRNA into 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 amount of template RNA added for each reaction was 1 µg. PCR cycler settings for cDNA amplification are shown in Table 4. Final PCR products were run on 1.2% agarose E-gels (Invitrogen, CA) for visualization of cDNA bands using Kodak UV/NIR image station.

### Table 4: PCR cycler settings for RT-PCR experiments

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (degrees Celsius)</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>Initial PCR Activation</td>
<td>95</td>
<td>15</td>
</tr>
<tr>
<td>3-Step PCR Cycling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>− Denaturation</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td>− Annealing</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>− Extension</td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>Number of Cycles - 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>10</td>
</tr>
</tbody>
</table>

### 3.5.3 Quantitative mIL-10 Transfection Studies with ELISA:

For quantitative analysis of mIL-10 gene expression caused by naked plasmid mIL-10, NiE, ME or GNP mIL-10 specific ELISA was performed. Towards this end, 200,000 J774A.1 cells were plated in different wells of six well plates. Following cell attachment, cells were treated with mIL-10 containing formulations
in serum free DMEM media for six hours. Following six hours, cell culture medium was completely replaced with fresh DMEM medium containing 10% FBS. Cell culture supernatant was collected at 12 hours, 24 hours, 48 hours, 72 hours and 96 hours time points and stored at -80°C.

Colorimetric sandwich ELISA plates for mIL-10 protein were purchased from R&D Systems (Minneapolis, MN). ELISA assay was performed as per the kit protocol [47]. Briefly, 50 µl of assay diluent and 50 µl of standard, sample or control were added to different wells. After gentle mixing, plates were covered with a plate sealer and incubated for two hours. After two hours solution from each well was aspirated and wells were washed 5 times with 1X wash buffer. After this step, 100 µl of secondary antibody conjugated with horse radish peroxidase was added to each well and incubated for two hours. After two hours, again contents from each well was aspirated and wells were washed 5-times with 1X wash buffer. Followed by this step, 100 µl of substrate solution containing hydrogen peroxide and tetramethyl benzidine was added into each well and incubated for 30 minutes protected from light. At the end of 30 minutes, stop solution containing diluted hydrochloric acid was added and absorbance at 450 nm with correction wavelength at 540 nm using BioTek Synergy HT (Winooski, VT) plate reader using KC4 software.

3.6 IL-10 gene transfection mediated suppression of pro-inflammatory cytokines

In order to evaluate IL-10 gene transfection mediated suppression of pro-inflammatory cytokines such as TNFα and IL1β, LPS stimulation studies were performed. Towards this end, 200,000 J774A.1 cells were plated in different wells of six well plates. Following cell attachment, cells were treated with mIL-10 containing formulations in serum free DMEM media for six hours. Following six hours, cell culture medium was completely replaced with fresh DMEM medium containing 10% FBS. Cell culture supernatant was collected at 12 hours, 24 hours and 48 hour
time points. Six hours prior to each time point, lipopolysaccharide (LPS) was added to each well at final concentration of 100 ng/ml in each well. RT PCR analysis was used for detection of TNFα and IL1β mRNA levels at each time point. TNFα and IL1β specific ELISA assay plates obtained from R&D systems (Minneapolis, MN) were used for detection of TNFα and IL1β protein levels in cell culture supernatant at each time point.

3.6.1 TNFα and IL1β RT-PCR Analysis: After completion of each time point, total cellular RNA was extracted from cells using High Pure RNA Isolation Kit (Roche, Germany) as per the kit protocol [45]. Quantification of isolated RNA was performed using NanoDrop® 2000c instrument (Thermo-Scientific, Delaware). The isolated RNA fractions were converted to final PCR products using selective primers for TNFα and IL1β and beta-actin using One Step RT-PCR kit (Qiagen, Valencia, CA) as per kit manufacturer protocol [46]. The forward and reverse primer sequences used for conversion of mRNA into cDNA for TNFα were 5’-CATGAGCACAGAAAGCATGATC-3’ and 5’-CCTTCTCCAGCTGGAAGACT-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. The amount of template RNA added for each reaction was 1µg. PCR cycler settings for cDNA amplification are shown in Table 4. Final PCR products were run on 1.2% agarose E-gels® (Invitrogen, Carlsbad, CA) for visualization of cDNA bands using Kodak UV/NIR image station.

3.6.2 TNFα and IL1β ELISA Analysis: ELISA assays were performed as per the kit protocols [48, 49]. Briefly, 50 µl of assay diluent and 50 µl of standard, sample or control were added to different wells in both plates. After gentle mixing, plates were covered with a plate sealer and incubated for two hours. After two hours solution from each well was aspirated and wells were washed 5 times with 1x wash buffer. After this step, 100 µl of secondary antibody conjugated with horse-radish peroxidase was added to each well and incubated for two hours.
After two hours, again contents from each well was aspirated and wells were washed 5 times with 1X wash buffer. Followed by this step, 100 µl of substrate solution containing hydrogen peroxide and tetramethyl benzidine was added into each well and incubated for 30 minutes protected from light. At the end of 30 minutes, stop solution containing diluted hydrochloric acid was added to both the plates and absorbance at 450 nm with correction wavelength at 540 nm using BioTek Synergy HT plate reader using KC4 software.
4. RESULTS AND DISCUSSION

4.1 Nanoparticle Characterization

The control and plasmid DNA encapsulated nanoparticle size and surface charge (zeta potential) values were measured using a Malvern's Zetasizer® either before or after ultracentrifugation. The results shown in Table 5 show that the particle size was in the range of 160 nm to 170 nm in diameter and the zeta potential values negative. As the isoelectric point of type B gelatin is in the range of 4.5 to 5.5, the formed nanoparticles will have a net negative charge at pH 7.4. Plasmid DNA is physically encapsulated in a hydrogel type matrix of type B gelatin biopolymer in contrast to electrostatic condensation by positively charged lipids and polymers [31].

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Gelatin Particles</td>
<td>164.3 ± 5.0*</td>
<td>-10.7 ± 0.12</td>
</tr>
<tr>
<td>EGFP-N1 Plasmid-Containing Nanoparticles</td>
<td>166.0 ± 5.6</td>
<td>-11.2 ± 1.35</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation (n=3)

4.2 Nanoparticles-in-Emulsion Formulation and Stability Studies

Stability of the nanoparticles-in-emulsion (NiE) tri-phasic system was evaluated using accelerated centrifugation and evaluation of phase separation under different conditions. When the multiple emulsion or NiE was centrifuged at up to 4,000 rpm for 1 hour, 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 by fluorescence
microscopy of rhodamine-labeled dextran either when administered directly into the W/O primary emulsion or upon nanoparticle-encapsulation.

Figure 2: Bright-field and fluorescent microscopy images of NiE (A&B) and ME (C&D) showing stable non-leaky multiple emulsion globules of 5.0 um or less diameter. Original magnification was 60X.

The results of preliminary stability studies showed that the both W/O/W and NiE formulations did not phase separate upon storage and even after accelerated centrifugation cycle. In addition, as shown in Figure 2, the internalize rhodamine-dextran in the inner aqueous phase or when encapsulated in gelatin nanoparticles did not release their content in the external aqueous phase even after 7 days of incubation at 4°C.
4.3 Plasmid DNA Encapsulation and Stability Studies

EGFP-N1 plasmid DNA loading efficiency was calculated as percentage of plasmid loaded compared to plasmid added initially. Results shown in Table 6 show that plasmid loading efficiency was increased, when plasmid was protected by encapsulation in GNP in NiE formulation compared to ME formulation in which unprotected naked plasmid was present in the internal phase of ME. Later a separate group of blank emulsion formulations were terminally spiked with EGFP-N1 plasmid DNA after completion of both steps of emulsification process. Then similar plasmid extraction procedure was carried out. Amount of plasmid assayed from these experiments was found to be 99.16% ± 0.35 of that added terminally. This result indicated that decrease in plasmid loading efficiency was might be due to high shear homogenization process.

Table 6: Plasmid DNA encapsulation efficiency in type B gelatin particles and in nanoparticles-in-emulsion formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>DNA Loading Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked plasmid in W/O/W multiple emulsion</td>
<td>54.4 ± 3.0</td>
</tr>
<tr>
<td>Plasmid encapsulated in type B gelatin nanoparticles</td>
<td>99.0 ± 0.5</td>
</tr>
<tr>
<td>Plasmid encapsulated in gelatin nanoparticles-in-emulsion</td>
<td>70.3 ± 2.2*</td>
</tr>
</tbody>
</table>

*Mean ± S.E (n=3)

For analysis for plasmid DNA stability, agarose gel electrophoresis was used. As shown in Figure 3, a single band of pEGFP-N1 of 4.7kb size was observed for all three formulations indicating that EGFP-N1 plasmid DNA retained its stability throughout the formulation process. Wells loaded with control formulations did not show any band.
Figure 3: Determination of EGFP-N1 plasmid DNA stability by agarose gel electrophoresis.

Lane 1: 2-10kb super-coiled 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 ME formulation.
Lane 5: EGFP-N1 plasmid DNA isolated from gelatin nanoparticles.
Lane 6: Blank gelatin nanoparticles.
Lane 7: Pure 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.

4.4 Uptake and Cellular Internalization in Macrophages

The microscopy images in Figure 4 shows that the rhodamine-dextran containing W/O/W emulsion and NiE formulations were rapidly internalized in murine alveolar macrophages J774A.1 cells. As time progressed, an increase in fluorescence intensity was observed, which became maximum after 120 minutes of exposure.
Figure 4: Bright-field and fluorescent images in figure (A,B), (C,D) and (E,F) shows cellular uptake of Rhodamine dextran containing ME formulation by J774A.1 cells at 60 minutes, 90 minutes and 120 minutes time points respectively. Bright field and fluorescent images in figure (G,H), (I,J) and (K,L) shows cellular uptake of Rhodamine dextran (70,000 MW) containing NiE formulation by J774A.1 cells at 60 minutes, 90 minutes and 120 minutes time points respectively. Original magnification was 40x.

Furthermore, DNA trafficking studies were performed in order to assess ability of different formulations for delivery of DNA within the nuclei of the cells. Results of these studies are shown in Figure 5. Cellular internalization of GNP, ME and NiE was observed starting 30 minute time point post formulation treatment. At the initial 30 minute time point, particles were further away from cell nuclei. Starting from 2 and 4 hour time point post treatment with different formulations, the distance between particles and nuclei was reduced and also co-localization of green and blue colors was observed. This indicated that DNA delivered through GNP, ME or NiE formulations was able to reach the nuclear compartment of the cell within two to four hours of formulation incubation. Along with increase in intracellular uptake of formulations with time, an increase in nuclear co-localization of DNA with time was also observed indicating particle movement from periphery towards cell nucleus. At 6 hour time-point, the red color was localized
near the perinuclear space, while green color was largely co-localized with blue color. These results indicate that GNP, ME and NiE were capable of delivering DNA within nuclear compartment of the cells.

**Figure 5:** Fluorescent microscopy images showing localization of DNA (green), GNP (red) and Nucleus (blue) within J774A.1 cells at 30 minutes, 2 hours and 6 hours time points post-treatment with either NiE, ME or GNP formulations. Original magnification was 60x.

### 4.5 Reporter (EGFP) Gene Transfection Studies

#### 4.5.1 Qualitative Transgene Expression Analysis:
Fluorescence microscopy was used for qualitative determination of EGFP-N1 gene transfection. Microscopy images in Figure 6 shows that maximum gene transfection was observed in cells treated with NiE formulations followed by ME and GNP formulations. Maximum green fluorescence was observed in cells treated with NiE formulation starting 24 hour post treatment with sustained levels at day 2, 3 and 4 followed by a decline at day 5 and 6. These results indicate that NiE formulation was better compared to ME or GNP formulations in causing gene transfection since NiE formulation offer
more protection to plasmid DNA during formulation resulting in higher loading efficiency of plasmid DNA per ml of formulation as well as during intracellular transport of plasmid DNA in endosomal/lysozomal compartments.

**Figure 6:** Evaluation of qualitative gene transfection by fluorescent microscopy

(a): Differential interference contrast and fluorescent microscopy images showing green fluorescent protein expression of in J774A.1 cells 24 hour post-treatment with EGFP-N1 plasmid DNA complexed with Lipofectin® (A,B), or encapsulated in NiE (C,D), ME (E,F) and GNP (G,H). Original magnification was 40X

(b): Differential interference contrast and fluorescent microscopy images showing green fluorescent protein expression of in J774A.1 cells 48 hours post-treatment with EGFP-N1 plasmid DNA complexed with Lipofectin® (A,B), or encapsulated in NiE (C,D), ME (E,F) and GNP (G,H). Original magnification was 40X
(c): Differential interference contrast and fluorescent microscopy images showing green fluorescent protein expression of in J774A.1 cells 72 hours post-treatment with EGFP-N1 plasmid DNA complexed with Lipofectin® (A,B), or encapsulated in NiE (C,D), ME (E,F) and GNP (G,H). Original magnification was 40X

(d): Differential interference contrast and fluorescent microscopy images showing green fluorescent protein expression of in J774A.1 cells 96 hours post-treatment with EGFP-N1 plasmid DNA complexed with Lipofectin® (A,B), or encapsulated in NiE (C,D), ME (E,F) and GNP (G,H). Original magnification was 40X
(e): Differential interference contrast and fluorescent microscopy images showing green fluorescent protein expression of in J774A.1 cells 120 hours post-treatment with EGFP-N1 plasmid DNA complexed with Lipofectin® (A,B), or encapsulated in NiE (C,D), ME (E,F) and GNP (G,H). Original magnification was 40X

(f): Differential interference contrast and fluorescent microscopy images showing green fluorescent protein expression of in J774A.1 cells 144 hours post-treatment with EGFP-N1 plasmid DNA complexed with Lipofectin® (A,B), or encapsulated in NiE (C,D), ME (E,F) and GNP (G,H). Original magnification was 40X
4.5.2 Quantitative Transgene Expression Studies: Quantitative evaluation of EGFP-N1 gene transfection was performed using EGFP-N1 ELISA. The results shown in Figure 7 show that NiE formulation was the most effective in causing gene transfection followed by ME and GNP formulations. NiE showed highest levels of gene transfection 24 hour post transfection with sustained levels till day 4 followed by a decline at day 5 and 6.

![Quantitative GFP Transfection](image)

**Figure 7**: Quantitative green fluorescent protein (GFP) expression by ELISA showing transgene expression with EGFP-N1 plasmid DNA encapsulated in GNP, ME, and NiE as well as complexed with Lipofectin®. Highest levels of GFP expression was observed with NiE formulation relative to all other tested.

4.6 mIL-10 Therapeutic Gene Transfection Studies

4.6.1 Isolation of mIL-10 Plasmid DNA from Transformed E. coli: For determination of purity of plasmid extraction, A260/A280 ratio was determined. A260/280 values for all extractions was within the window of 1.9 to 2.0 which indicated plasmid extracts contained none or negligible protein contamination. Further, when plasmid extracts were run on 1.2 % agarose E-gels®, an intact DNA band of size approximately 3.7 kilobase pair was observed. In order to determine presence of IL-10 transgene in the extracted plasmid, plasmid extract was digested with two restriction enzymes Nhel and Ncol. When digested plasmid was run on 1.2% agarose
E-gel, a band at approximately 500 bps was observed which co-related with size of IL-10 transgene as described by IL-10 transformed *E. coli* supplier Figure 8. These results show that we were able to isolate and purify mIL-10 plasmid successfully free from protein or genomic nucleic acid impurities in stable form.

Figure 8: (A) Agarose gel electrophoresis showing intact plasmid of approximately 3.7 kilo base pairs (lane 2). Lane 4 shows presence of a DNA fragment of approximately 500bps in size correlating with size of IL-10 transgene in the plasmid vector. (B) mIL-10 plasmid constructs.

**Lane 1:** Supercoiled DNA ladder 2-10 kb.  
**Lane 2:** Undigested plasmid IL-10.  
**Lane 3:** 100 – 1500 bp DNA ladder.  
**Lane 4:** IL-10 plasmid digested with Nco-I and Nhe-I restriction enzymes.

4.5.2 RT-PCR Analysis for mIL-10 mRNA Expression: Results obtained from IL-10 RT-PCR showed highest expression of IL-10 mRNA in cells treated with NiE formulation followed by
ME and GNP formulations at different time points. Compared to Lipofectamine, a standard transfecting reagent, NiE showed higher IL-10 mRNA expression. Results are shown in Figure 9.

![IL-10 RT PCR - Gel Image Analysis](image)

**Figure 9:** Qualitative IL-10 mRNA expression by RT-PCR in J774A.1 cells. NiE formulations showed higher mIL-10 transgene transcript expression relative to all other tested.

**3.5.3 Quantitative mIL-10 Gene Transfection Studies with ELISA:** Results obtained from mIL-10 ELISA correlated with IL-10 RT-PCR experiment where NiE formulation showed highest gene transfection efficiency followed by ME and GNP. ELISA results are graphed in Figure 10.
Figure 10: Quantitative IL-10 protein expression by ELISA showing transgene expression with mIL-10 plasmid DNA encapsulated in GNP, ME, and NiE as well as complexed with Lipofectin®. Highest levels of mIL10 expression were observed with NiE formulation relative to all other tested.

4.7 In Vitro Anti-Inflammatory Activity of Expressed mIL-10

4.7.1 TNFα and IL1β RT-PCR Analysis: LPS triggered expression and release of pro-inflammatory cytokines was evaluated using RT-PCR analysis. At the end of 12 hour and 48 hour time points, levels of TNFα and IL1β in cells pretreated with IL-10 gene containing formulations was lower compared to untreated cells. Among the different formulations tested, NiE formulation which was most efficient in causing mIL-10 gene transfection showed highest suppression of TNFα and IL1β gene transcription followed by ME and GNP formulations. Results of this study are shown in Figure 11.
**Figure 11:** RT-PCR analysis for TNFα and IL1β mRNA in untreated cells (lane 3) or treated with naked plasmid (lane 4), mIL10 plasmid complexed with Lipofectin® (lane 5), mIL10 plasmid in NiE (lane 6), mIL10 plasmid in ME (lane 7) or mIL10 plasmid in GNP (lane 8) at 24 hour and 48 hour time points after LPS stimulation.
4.7.2 TNFα and IL1β Expression Analysis by ELISA: ELISA for TNFα and IL1β was performed in order to quantitate levels of these cytokines in cell culture supernatant at different time points following LPS stimulation on IL-10 transfected J774A.1 cells. The results (figure 12 and 13) showed highest suppression of both TNFα and IL1β in cells treated with NiE followed by ME and GNP formulations. These results indicate that IL-10 plasmid delivered through NiE has therapeutic activity where it can suppress expression of pro inflammatory cytokines such as TNFα and IL1β.

**Figure 12:** Quantitative TNFα protein expression by ELISA showing IL-10 transfection mediated suppression of TNFα in either untreated J774A.1 cells or J774A.1 cells transfected with mIL-10 containing NiE, ME, GNP, Lipofectin® or naked mIL-10 plasmid.

**Figure 13:** Quantitative IL1β protein expression by ELISA showing IL-10 transfection mediated suppression of IL1β in either untreated J774A.1 cells or J774A.1 cells treated with mIL-10 containing NiE, ME, GNP, Lipofectin or naked mIL-10 plasmid.
5. CONCLUSIONS

The results of this study show that DNA-containing solid gelatin nanoparticles can be encapsulated in the innermost aqueous phase of the W/O/W multiple emulsions to form NiE formulations. EGFP-N1 or mIL-10 plasmid DNA-loaded NiE formulations were capable of producing sustained gene transfection in J774A.1 murine adherent alveolar macrophage cell lines. Moreover, it was found that plasmid EGFP-N1 or mIL-10 encapsulated in NiE had superior gene transfection efficiency compared to plasmid EGFP-N1 or mIL-10 encapsulated in ME or GNP or complexed with Lipofectin®, a cationic-lipid based transfection reagent. Furthermore, it was confirmed that mIL-10 delivered through NiE was capable of down regulating levels of proinflammatory cytokines TNFα and IL1β in LPS stimulated J774A.1 cells.
6. REFERENCES

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