HYALURONIC-ACID BASED NANOCARRIERS FOR RNAi THERAPY OF ADVANCED SEPSIS

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by
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Abstract

Ever since the first representation of RNAi phenomenon in 1998, its ride has been quite complicated. If handled carefully, RNAi could have the potential of transforming into a therapeutic which can apply in a wide range of diseases from viral to cancer. The use of a powerful therapeutic tool like siRNA therapeutics has faced many barriers, like: molecule stability, specific delivery to desired cells and tissues, triggering of innate immune response, and effects achieved off the desired site. Theoretically, siRNA can be used to silence any gene, therefore is a potential therapeutic to many genetic diseases, and not only. However, given the enormous genetic variation between species RNAi becomes a challenge. Sepsis syndrome is one of those diseases that quickly progresses over time. There is a demanding need in finding a therapy for advanced sepsis syndrome, a fast-running progressive disease. The main mediators of inflammation in general and sepsis in particular like, TNF-α and IL1 play an important role in triggering a spillover of a flood of cytokines leading to sepsis condition. Anti-TNF-α therapies have been applied in inflammatory diseases and in sepsis in a long time, but despite the promising results of the in vivo work the translation to humans has been difficult. From its role prospects, TNF-α gene is a tricky target to modulate, and siRNA therapy offers transitory effect instead of an enduring effect over this target. We developed a nanosized delivery system, made of modified hyaluronic acid polymer encapsulating cholesterol modified siRNA in its core, to enable the siRNA supply in the macrophages` cytoplasm. The particle we developed was characterized and a size of nanometer range was determined, which intact the siRNA through charge and hydrophobic interaction. The main goal of this thesis work was to develop a therapy for sepsis utilizing RNAi to target TNF-α, based on biomarkers identification to follow on the disease progression. One important goal achieved was the creation of a multimodal delivery
system, to overcome the long known obstacle of siRNA delivery in cells cytoplasm. More specifically, targeting macrophage cells is a complicated task. Despite their ability to take up quickly, they also possess the high capability of degrading the engulfed materials. Nanotechnology principles are very helpful in generating delivery vehicles with unique physicochemical and biological properties. As a nanosized system, the formulation offered by this thesis work represents a combinatorial approach that contains poly(ethylene imine) (PEI) modified hyaluronic acid polymer in combination with lipid modified polymer. A poly(ethylene glycol) (PEG)-modified polymer is used as a component of the formulation to provide more stability given the negative charge of the siRNA. On one hand, the positively charged PEI plays an important role in encapsulating the siRNA through charge interaction, while on the other hand it contributes a proton sponge effect within the cells that facilitates the siRNA delivery into cytoplasm after escaping the late endosome. The chemical modification of the siRNA, by addition of a cholesterol molecule, and addition of lipid modified hyaluronic acid polymer, lead to improvement of encapsulation efficacy, while enhancing the TNF-α silencing efficacy in peritoneal macrophages. The silencing efficacy was supported by the biodistribution data in vivo, which showed efficacious siRNA uptake preferably in peritoneal macrophages, as compared to other organs tissues in mice. Septic mice treated with anti- TNF-α siRNA nanoparticle showed a significantly improved survival rate, as compared to septic mice treated with scramble siRNA nanoparticle, in a LPS developed model of sepsis. Thus, the novel multifunctional nanoparticle encapsulating siRNA, offers an alternate, safe, and effective siRNA delivery system to treat sepsis, and in more general terms, the inflammatory diseases.
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RESEARCH OBJECTIVE AND SPECIFIC AIMS

1. Research Objectives

Sepsis follows from the systemic inflammatory response syndrome, and its severity is positively correlated with the host immune response. By definition, severe sepsis is associated with organ dysfunction which can be specific, or multiple. Systemic hypotension and tissue hypo-perfusion add up to severe sepsis, which leads to a septic shock condition. At this point, the outcome for the patients is poor: in the United States the incidence of death among patients with septic shock is as high as 40% [1], [2], [3]. Sepsis and septic shock occur when an antigen triggers anti-inflammatory response resulting in a cascade of cytokine release. The Tumor Necrosis Factor (TNF-\(\alpha\)) is known to play a key role in these conditions, but the levels of other anti-inflammatory and pro-inflammatory cytokines also undergo profound changes. The complexity of sepsis indicates a need for therapeutic agents that are specific to the stage of disease [4].

*The central hypothesis of this proposal is that animals with a severe sepsis condition that are treated with an anti-TNF-\(\alpha\) short interfering ribonucleic acid (siRNA) drug, will experience significant survival benefit, relative to untreated animals at the same stage of the disease.*

*Our main objective is to develop a nanoparticle delivery system for *in-vivo* targeting of the TNF-\(\alpha\) gene in activated macrophages in which a sepsis condition has been induced; we argue that such treatment will result in a positive survival outcome. To achieve this objective, we will use the following tools: (i) a hyaluronic acid (HA) functionalized polymer to deliver the siRNA in activated macrophages in the local area of inflammation induction; (ii) a HA*
functionalized polymer that can escape the endosome compartment, delivers siRNA in the cytosol, and results in specific silencing.

2. Specific Aims

To achieve our objective, we will address the following aims:

Specific Aim 1: To prepare and characterize HA-based self-assembling nanosystems for encapsulating siRNA duplexes.

Prepare an HA-based polymer that is modified with functional groups, and use a RiboGreen assay to evaluate its encapsulation efficiency with siRNA.

We propose to evaluate the TNF-α gene silencing efficacy for siRNAs of different chemical modifications. RT-qPCR will be used to evaluate the silencing efficiency.

And finally, we plan to optimize the nanoparticle formulation design by chemically functionalizing its two components: the HA polymer and siRNA.

Specific Aim 2: To evaluate in vitro the efficiency of delivery of the nanosystem and of gene silencing, and to measure the anti-inflammatory response.

We propose to use J774A.1 macrophages and confocal microscopy to evaluate the time course and efficiency of nanoparticle uptake by the cells, using nanoparticles made of fluorescently labeled polymer and siRNA.

RT-qPCR will be used to quantitatively and qualitatively evaluate the efficacy of TNF-α gene silencing.
FACS analysis will be applied to assess CD44 receptor expression on the J774A.1 macrophages; and we will add Lipopolysaccharides (LPS) to study the potential of the macrophages to mount an anti-inflammatory response.

**Specific Aim 3: To develop a mouse model of sepsis.**

We propose to develop a mouse model of endotoxic sepsis.

We will evaluate the cytokine profile in blood with respect to progressive stages of disease.

We will assess the efficacy of the siRNA formulated with the HA modified polymer by measuring the levels of TNF-α in the blood, and other cytokines relative to it.

**Specific Aim 4: To evaluate the efficacy of gene silencing *in-vivo*.**

A fluorescently labeled nanoparticle made of siRNA encapsulated in HA polymer will be used to assess its biodistribution.

We will measure changes in the levels of TNF-α cytokine in blood serum, and liver and spleen macrophages, before and after treatment with therapeutic siRNA encapsulated in the HA polymer.

**Specific Aim 5: To evaluate the therapeutic efficacy and safety of nanoparticle system *in-vivo*.**
Liver tissue will be histologically analyzed or immune response will be evaluated to check for acute toxicity. The cytokine profile in the blood will be evaluated.

Changes in cytokine profile in the blood will be used to assess sepsis progression before and after treatment with siRNA-HA polymer nanoparticle.

The survival benefit will be evaluated in treated animals versus untreated animals, using appropriate statistical testing.

This project is innovative, because it will use a biodegradable polymer developed in our laboratory based on its targeting specificity. To the best of our knowledge, inflammatory disease and rheumatoid arthritis have been studied with the use of siRNA therapies, but sepsis has not. We aim to develop an anti TNF-α drug, using RNAi which if used in advanced phases of sepsis, will restore the balance between SIRS and CARS and will increase the survival rate.
CHAPTER 1
SEPSIS, TUMOR NECROSIS FACTOR-ALPHA, AND RNA INTERFERENCE

1. Sepsis and Triggers of Inflammatory Response

Sepsis is a medical condition caused by a severe body response to infection. In advanced sepsis the patient dies from his body inflammatory response to infection, rather than from the infection itself [5].

1.1 Infection-induced inflammatory response and escalation to a “cytokine storm”

In 1972 Lewis Thomas simplified the notion of uncontrolled inflammatory response when he named the microorganisms “….bystanders”, and host organism “….more in danger from its own arsenal for fighting off bacteria… than from the invaders”. The invading stimuli hold responsible for macrophage activation and host immune stimulation. This immune stimulation is beneficial during the early phase of response, else called Systemic Inflammatory Response Syndrome (SIRS)[1], [2], [3]. The immune stimulation at early phases of SIRS is characterized by the cytokine release, which can become offensive in the later phases of sepsis when both pro-inflammatory and anti-inflammatory response become severe. The crosstalk between pro and anti-inflammatory responses generates increasingly high levels of cytokines, which has been defined as “cytokine storm” to explain the devastating impact in tissue and organ level. As the name suggests, the “cytokine storm” is difficult to refrain and leads to organ dysfunction and lethal outcome [6] [7].

Unlike the theory of uncontrolled hyper-inflammation postulated by Lewis Thomas [8], which led to anti-inflammatory therapies, the Munford and Pugin postulated that the immune cells have a dual role: pathogenic and protective [9], [10]. According to these scientists, the blockage of the mediators harms the host’s immune system. If it was true, this other postulate would be a
possible explanation for why the pro or anti-inflammatory therapies have not been successfully effective.

### 1.2 Balance between SIRS and CARS during inflammation and sepsis

The mechanisms of immune system response to an insult are very well organized, consisting of fragile balances of start and stop “switches”. In the case of infection the systemic inflammatory response is responsible for starting the host inflammatory response to contain the infection. Mediators such as TNF-alpha, interleukins IL-1, IL-6, IL-12 start the signaling cascade, which in an effective response is countered by the compensatory anti-inflammatory response syndrome (CARS) mediated by IL-1ra, IL-4, IL-10 and IL-13. A balanced response between SIRS and CARS establishes very often during viral and bacterial infections, resulting in host recovery sometimes without having symptoms [11]. Due to excessive inflammation, SIRS-CARS balance is lost during severe inflammation or development of septic shock, leading to immune suppression and organ damage [4]. It is important to know the cytokine storm pathology, which starts at a local site, but soon after warns the host through systemic circulation.

The systemic response is associated with compensatory inflammation, which if not restored leads to tissue persistent organ dysfunction or healing with fibrosis (Figure 1)[12].

**Figure 1.** Schematic that shows sepsis progression through 5 phases. Phase 1 represents inflammatory response limited at the site of infection; Phase 2
represents spillover of cytokines into the systemic circulation; Phase 3 represents progression to SIRS; Phase 4 represents development of CARS; Phase 5 represents the consequences of immunologic antagonism. The number of arrows illustrates the intensity of the event.

It is known that the acute response is characterized by the cytokines TNF and IL-1 and chemotactic cytokines IL-8 and MCP-1, followed by a sustained increase in IL-6. Although the pro-inflammatory cytokines are necessary to launch an effective process against the infection, their excess production is associated with multiple organ dysfunction and mortality [13]. Unlike the pro-inflammatory response, the anti-inflammatory response is critical in down-regulating the inflammatory response. The immunosuppression and inability of the host to clear the infection are typical features in septic patients, which change rapidly over time. An increase in levels of the anti-inflammatory cytokine IL-10 testifies that the host system has started the attempts to control the systemic response, increasing alongside sepsis severity [6], [4], [14]. Besides the cytokine levels, knowledge on the complex cytokine overlapped network are with particular importance when pro-inflammatory or anti-inflammatory therapeutic agents are used. Pro-inflammatory overexpression as well as immune deficiency followed from anti-inflammatory response, might be life-saving if targeted at the deserved time to prevent a lethal immune paralysis [10]. A summary of cytokines and mediators in sepsis is shown in Table 1 [15].

Table 1. Summary of cytokines released during inflammation and sepsis, and their respective roles

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Role in Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>Act in hematopoiesis, control the production, differentiation, and function of 2</td>
</tr>
<tr>
<td>granulocyte-colony stimulating factor</td>
<td>related white cell populations of the blood, the granulocytes and the monocytes-macrophages</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Regulate cell functions, differentiation of macrophages and granulocytes, dendritic</td>
</tr>
<tr>
<td>granulocyte-macrophage colony-stimulating factor</td>
<td>cell development and the maintenance of homeostasis</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Coordinates a diverse array of cellular programs through transcriptional regulation</td>
</tr>
<tr>
<td>Interferon- gamma</td>
<td>of immunologically relevant genes; pro-inflammatory</td>
</tr>
<tr>
<td>IL-1</td>
<td>Proximal cytokine. Controls different cellular functions, proliferation, differentiation and cell survival. IL-1 is responsible for the production of inflammation, as well as the promotion of fever and sepsis. IL-1β mediates</td>
</tr>
<tr>
<td>Cytokine</td>
<td>Function and Characteristics</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>IL-2</td>
<td>Produced by the T_{H}1 lymphocytes; enhances cellular inflammation</td>
</tr>
<tr>
<td>IL-4</td>
<td>Produced by the T_{H} cells exercising anti-inflammatory action</td>
</tr>
<tr>
<td>IL-5</td>
<td>Pro-inflammatory cytokine</td>
</tr>
<tr>
<td>IL-6</td>
<td>Distal cytokine. Serves as both a marker and a mediator for the severity of sepsis. Lack of it does not affect mortality due to sepsis.</td>
</tr>
<tr>
<td>IL-7</td>
<td>Potent antiapoptotic cytokine that is essential for lymphocyte survival and expansion. IL-7 induces proliferation of naïve CD4 and CD8 T cells potentially replenishing the loss of naïve T cells that occurs during sepsis</td>
</tr>
<tr>
<td>IL-9</td>
<td>T cell-derived cytokine implicated in the response to inflammatory processes; may be implicated in induction of IL-10</td>
</tr>
<tr>
<td>IL-10</td>
<td>Anti-inflammatory cytokine with inhibitory effect on TNF-α, IL-6, and IL-1</td>
</tr>
<tr>
<td>IL-12</td>
<td>Promote innate immunity; facilitates T-helper (Th1)-cell responses, and IFN-γ production. Consists of two covalently linked subunits of 35 (p35) and 40 kD (p40). Both chains must be expressed simultaneously by a cell to generate the biologically active heterodimer</td>
</tr>
<tr>
<td>IL-13</td>
<td>Exerts potent anti-inflammatory properties during sepsis</td>
</tr>
<tr>
<td>IL-17</td>
<td>Proinflammatory cytokine produced by a variety of cells including CD4^+ Th17 cells, CD8^+ T cells, neutrophils and NK cells</td>
</tr>
<tr>
<td>IP-10</td>
<td>Pro-inflammatory chemokine</td>
</tr>
<tr>
<td>KC</td>
<td>Chemoattractant, activates neutrophils, stimulates neutrophil influx into site of infection</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>MIP-1a</td>
<td>Is generated by macrophages in response to inflammation</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>Exists in MIP-1a, MIP-1b, MIP-2 forms, all contribute to inflammation response</td>
</tr>
<tr>
<td>MIP-2</td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>Secreted by the normal T cells, chemotactic for monocytes, eosinophils, basophils, CD4^+ T lymphocytes; elevated levels in sepsis</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Primary mediator of inflammation through signaling of cytokine production, with large implication in sepsis</td>
</tr>
</tbody>
</table>

### 1.3 Uncontrolled inflammation leading to immune suppression and organ dysfunction

It is believed that a balanced inflammatory response is necessary to contain an invasive infection, and when the balance is lost, sepsis and septic shock are developed. In a long time tissue hypoxia and hypoxemia have been considered to cause multiple organ dysfunction syndrome (MODS) in critically ill patients [16], [17]. The future challenge is to reveal measurable critical levels of host responses that still have the benefit of fighting the menacing trigger, while supporting host survival [17].

### 2 TNF-alpha as an Important Mediator in Sepsis

#### 2.1 Targeting TNF-alpha
Septicemia and sepsis together are fatal ranked as the 11th leading cause of death in 2010. During the year 2008, sepsis mortality rose from 28 to 48% in affected patients [4], [18]. Survivors of severe sepsis are highly likely to have permanent organ damage and physical disability. Despite the continuous investment and the development of intensive care units, the inability to lower mortality figures is still apparent [19]. Successful treatment of sepsis requires aggressive therapy in the intensive care unit during the early phases, followed by a long period of patient hospitalization. Clinically proven human therapies such as use anti-TNF-α mono-clonal antibodies were developed based on a better understanding of mechanisms of the disease; however this therapy is facing debating with regard to its survival benefits. TNF-α production is exaggerated during sepsis and immune regulatory factors are not able to prevent its systemic toxic effect. From previous studies it is known that this cytokine is a leading mediator of the inflammatory response during sepsis [3]. Increases in levels of the pro-inflammatory cytokine TNF-α subsequent to challenge with LPS in both animals and humans led to recognition of this factor as a therapeutic target. Mono-clonal anti-TNFα therapy against sepsis has been beneficial in cases where the severity of underlying disease is predetermined. To the best of our knowledge, there have been efforts in treating rheumatoid arthritis and bowel disease with use of RNAi therapeutics. Effective silencing of TNF-α expression \textit{in vivo} has been reported with use of liposomal nanoparticles for siRNA delivery in \textit{arthritis and inflammatory bowel disease} [20]. Based on these findings, we propose to develop an RNAi therapy for treating subjects with severe sepsis.

\subsection{2.2 RNA interference mechanism of action}

The development of RNA interference (RNAi) technology to treat diseases is considered to be a major technical breakthrough for many aspects of biology and medicine. The phenomenon
of RNAi was described in 1998 when Andrew Fire and Craig Mello, elucidated the requirements for efficient delivery of siRNAs to induce down-regulation of gene expression in *Caenorhabditis elegans*. Three years later in 2001, Tuschl, *et al.* established that RNAi is indeed triggered in mammalian cells by 19 and 21 nucleotide siRNAs that consist of 2 overhangs on the 3’ ends. Since its discovery, RNAi has evolved into a critical tool for research in molecular biology, and its use has been rapidly translated from bench top to the clinical trials [21]. The RNAi mechanism acts at the post-transcriptional level and the terms “RNAi” and “post-transcriptional gene expression silencing” are used interchangeably. RNAi is an endogenous process that affects the control of gene expression. Double stranded RNAs (dsRNAs) comprising 299 base pairs are processed to form siRNAs of 21 or 22 base pairs. If the anti-sense strand, is fully complementary to the (mRNA) fragment of a gene, it effectively mediates gene expression knockdown for that gene: this finding led to the demonstration *in vitro* that *synthetic* siRNAs can be designed with one sequence that is complementary to the mRNA to silence the gene of interest in cultured mammalian cells [22]. Once it is in cytoplasm, the exogenously introduced synthetic siRNA becomes incorporated into a protein silencing complex called the RNA-Induced Silencing Complex (RISC). Argonaut 2, a key component of the RISC unwinds the sense and antisense strands of siRNA. The sense strand is cleaved off, while the antisense strand selectively finds the perfect complementary mRNA fragment and cleaves it, stopping the translation step (figure 2).
Figure 2. The mechanism of RNA interference. Long double-stranded RNA (dsRNA) enters the cytoplasm and gets cleaved into small interfering RNAs (siRNAs) by the Dicer enzyme. The siRNA can be delivered into the cell directly or via a carrier. The anti-sense strand of the siRNA gets incorporated onto the RNA-induced silencing complex (RISC), is unwound by the protein Argonaut 2 (AGO2) and remains intact; the sense strand on the other hand gets cleaved. RISC is thus activated, finds the complementary mRNA, binds to it and cleaves it, which in turn silences gene expression. The RISC-siRNA complex is recycled and reused for additional cleavage of the identical mRNA [23].

It has been hypothesized that therapeutic effect is ensured by the activated RISC complex, this moves to destroy other targets, and thus promotes gene silencing. This process of silencing continues for as long as the siRNA concentration in the cytoplasm is above a certain threshold, and could last for up to 7 days. Repeat dosing regimen is then needed to achieve perpetual effect [23].

2.3 Challenges to siRNA delivery in vivo

Direct siRNA delivery is possible to local tissues such as lung, but many tissues can only be accessed via systemic administration of the siRNA. Before reaching the cytoplasm of the target cell, the siRNA must bypass a series of hurdles including enzymatic degradation, reaction with
serum proteins, phagocytosis, and kidney filtration. Phagocytic cells are effective in removing the therapeutic complex, so the delivery vehicles must be designed to avoid opsonization and to prevent endonuclease cleavage of the siRNA [23]. Once inside the cell, the delivery particle must be able to leave the endosome and enter the cytoplasm without getting degraded in the lysosomes. However, although powerful algorithms have been developed for designing the active siRNA, administration of the molecule remain major challenges for therapeutic applications. Three interdependent phenomena particularly affect the delivery of siRNA: (i) stability of siRNA; (ii) penetration into cell; and (iii) targeting of specific tissue [21], [22], [24]. We aim to develop a stable nano-sized delivery system to treat sepsis: this system will have the ability to penetrate cell and to have a preferential distribution in the tissue(s) and organ(s) of interest. In theory, the RNAi mechanism can be set to silence any gene. This creates a broad range of applications. We argue that an effective RNAi strategy should overcome the susceptibility to nucleases, facilitate cell penetration, and target particular tissue(s) via use of specific ligands.

2.4 Nanoparticles as a delivery vehicle for siRNA

Non-modified siRNAs evoke an immune response by activation of Toll-like receptor 7 (TLR7). To avoid such stimulation, the sugar moiety on the RNA molecule is chemically modified, which in turn not only decreases the immune response, but increases the resistance of the siRNA to endonucleases and abolishes off-site effects. 2′-OMe modification, phosphorothioate modification on the backbone, and fluorine substitution on the 2′ position of the ribose are commonly used to avoid these effects [23]. Another approach to avoid immune stimulation involves the conjugation of small molecules to the 3′ end of the sense strand. For
example conjugation of a cholesterol molecule to the 3’ end of the sense strand reportedly is effective for improving biodistribution of the siRNA to the liver [25].

Delivery vehicles such as nanoparticles are used to minimize toxicity, while retaining the ability to selectively deliver therapeutic agents to the target tissues; to achieve these goals the vehicle must also bypass endosomal entrapment. Several approaches have been used to modify synthetic polymers with pH-sensitive functional groups that facilitate an escape from the endosome. These approaches include: (i) the use of pH-buffering agents and functional groups; (ii) destabilization of the endosomal membrane; (iii) photochemical disruption of endosomal membrane; (iv) use of functional groups that have membrane penetrating abilities [26]. Nanoparticles are delivered via the systemic circulation to target tissues and organs that are not accessible by the naked RNA. The most important benefits of using nanoparticles are however, the amelioration of surface properties; improvement of biodistribution to target organs such as liver, spleen, kidney and lungs; and prevention of immune system stimulation [23], [27]. Viral and nonviral vectors have been used for targeted delivery of siRNAs. Here we focus on use of nonviral vectors. Liposomes and lipoplexes have shown efficacy in overcoming the challenges of siRNA delivery. However, lipoplexes are not stable, and typically must be prepared immediately before use, which may not be practical for the clinical setting. Stable Nucleic Acid-Lipid Particles (SNALPs) reportedly dramatically enhance gene silencing potency of the apolipoprotein B gene in mice. Cationic lipid-containing systems are efficacious delivery vehicles due to their positive charge. Neutral liposomal systems are also efficient delivery vehicles due to their destabilizing effect on cell membrane. Conjugation of polymers like PEI and PEG with the siRNA molecule provides improved delivery into cells. Reportedly, the biocompatibility of hyaluronic acid (HA) makes it an attractive carrier for siRNA delivery and
has the advantage that can be modified with functional groups like PEI to improve specificity of targeting and siRNA encapsulation efficiency. However, the degree of modification of HA with PEI must retain a sufficient positive charge on the HA polymer. This positive charge electrostatically interacts with the negatively charged siRNA, this way avoiding the toxicity associated with the PEI itself [28]. Using a multifunctional approach that includes polyethylene imine modified hyaluronic acid polymer, we aim to target macrophages and knockdown their TNA-alpha levels. Based on the “cytokine storm” theory, TNF-alpha reduction can potentially lead to reduction of the signaling, and decrease the release of other cytokines down the steam of inflammation [29]. However, the intervention’s time management is of paramount importance. It is well-known that murine tissue macrophages express CD44 receptors, and that CD44 receptors and hyaluronic acid are target-ligand related to each-other. This is likely to facilitate the particle cell penetration in the cells where CD44 is expressed [30].

2.5 Current techniques for evaluation of in vivo siRNA delivery

Gene mutations can lead to enzyme deficiencies, missing ion channels, and uncontrolled cell growth. These phenomena are often considered to be candidates for gene therapy. Transgenic animals are an irreplaceable tool for evaluating the impact of the diseases with regard to biologically meaningful endpoints. However, note that environmental and genetic diversity are important factors that are not easy to stimulate in animal models. Human diseases that have been modeled (with varying degrees of success) with use of transgenic animals include, infection, neuro-degeneration, apoptosis, arteriosclerosis, and cancer [31]. Use of transgenic mice has grown along with imaging techniques field. Fluorescence imaging techniques are used for assessing gene silencing and tracking siRNA delivery in vivo: such imaging strategies require that either the carrier or the siRNA be labeled. Carriers in cell- culture studies have also been
labeled with quantum dots (QD). In tumor models, the use of MRI offers a noninvasive imaging methodology for \textit{in vivo} tracking by MRI of fluorescently labeled nanoparticles. Radionuclide-based (e.g., SPECT) and bioluminescence (e.g., PET) imaging are also suitable for determining biodistribution of siRNAs \textit{in vivo} and imaging with enhanced Green Fluorescent Protein (eGFP) is efficacious for evaluating long-term effects of RNAi. While these techniques are all still in various phases of development, they are considered to have enormous potential to uncover the unknowns of siRNA field [31].

2.6 \textbf{Animal models of sepsis}

Animal models of sepsis are based on use of three types of agents to trigger the disease: (i) administration of a toxin, like lipopolysaccharide (LPS) (ii) administration of a pathogen, like a gram negative bacteria (iii) alteration of the animal’s endogenous protective barrier, as by allowing bacterial translocation [4].

\textit{LPS- Induced Sepsis} model in animals, leads to rapid increases and high peak levels of pro-inflammatory cytokines compared to what has been reported for humans. However, when the amount of LPS administered is titrated to mimic the early stages of human disease, the model is especially useful for studying systemic and renal responses during the early phases of sepsis [32].

\textit{The Cecal Ligation and Puncture (CLP)} model is a polymicrobial model that leads to bacteremia followed by sepsis. Although not standardized, this model offers possibilities for studying the severity of the response by adjusting the length of the ligated cecum and the number of punctures. Similar to the CLP model is the colon ascendant stent peritonitis CASP model, wherein a stent is inserted to create a septic focus [1, 4].
**Bacterial Infusion or Instillation** models induce sepsis by introducing single pathogens in a controlled manner. Although the CLP and instillation models each have their own advantages and disadvantages, the instillation model offers greater reproducibility, and has been more readily transferred into larger animals, especially for the study of systemic or organ-specific hemodynamics [3],[4],[32].

Regardless of the model, there is a gap between animal models of sepsis and the human disease: the animal models lack use of intensive care facilities, raising the question of whether or not primate models are adequate surrogates for human sepsis. It is important to have an appropriate animal model for which findings can be translated into clinical practice [33].

### 2.7 Conclusions

Use of synthetic siRNA *in vivo* shows its therapeutic potential with regard to silencing genes in various diseases including hypercholesterolemia, liver cirrhosis, hepatitis B virus, human papilloma virus, and ovarian cancer [34]. Applications to other diseases, including those involving the silencing of genes for age-related macular degeneration, vascular endothelial growth factor, respiratory syncytial virus proprotein convertase subtilisin/kexin type 9, and transthyretin, are in clinical trial phases [23]. Figure 3 shows the diversity of targets and conditions which are currently being studied for RNAi therapeutics. This figure demonstrates that siRNA is being built as a platform, since theoretically can silence any gene. We aim to use the temporarily effect of the siRNA to target the TNF-alpha gene. This approach can provide positive impact in treating sepsis without permanently suppressing the host immune response.
Figure 3. Classification of siRNA drugs based on target tissue, condition and sponsoring company [35]
CHAPTER 2
SYNTHESIS OF MODIFIED HYALURONIC ACID DERIVATIVES, siRNA ENCAPSULATION, AND CHARACTERIZATION OF THE NANOPARTICLE FORMULATIONS

2.1 Introduction

As previously discussed in our Specific Aims section, the objective of utilizing a combinatorial HA polymer based nano-system, is to enhance the delivery of the siRNA into macrophages. The inherent properties of naked siRNA, such as mechanism of action, immune stimulation, instability in the bloodstream, and wide distribution in the living organism, require the necessity of an appropriate formulation that facilitates the delivery [36, 37]. We chose Hyaluronic Acid (HA) based polymers, because it represents an attractive approach due to the fact that it is a ligand for the CD44 receptors, a group of receptors that are fairly expressed on the myeloid cells, especially macrophages [38].

2.2 Materials and Methods

2.2.1 Rationale for the use of hyaluronic acid

Hyaluronic Acid (HA) is a non-sulfated glycosaminoglycan, which is a natural polysaccharide composed of disaccharide building blocks of D-glucuronic acid and N-acetyl-D-glucosamine linked by glycosidic linkage (Figure.4). The molecular weight of HA varies from 1000 to 10,000,000 Da. It has been used in various medical applications as it is biocompatible, biodegradable, non-toxic, and non-immunogenic. Cluster Determinant 44 (CD44) is the target receptor for HA; however other HA receptors are: 1) receptor for hyaluronate-mediated motility (RHAMM) and 2) lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1). HA conjugated with poly(ethylene imine) (PEI) is used to encapsulate siRNA molecules, based on the charge interaction between siRNA and PEI on the HA polymer [28], [38]. Our lab
synthesizes modified HA polymers with PEI, PEG, thiol, and alkyl functional groups. Figure 4, is a schematic image of the synthesis process of the HA modified polymers. The site of attack for enzymatic degradation of HA is its carboxylic group. Modification of the carboxylic group (Figure 4), contributes to the stability of the polymer to hyaluronidases.

**Figure 4. Synthesis of HA based functional macrostructures.** 1, Hyaluronic acid of 20kDa molecular weight; 2, HA conjugated to alkyl chains with the general formula H₂N(CH₂)ₙ (where n=3,4,5..); 3, HA conjugated to PEG of 2000 Da molecular weight; 4, HA conjugated to bifunctional fatty amines with the general formula NH₂(CH₂)ₙNH₂ (where n=4,5..); 5, HA conjugated to cysteamine and reduced to free thiol; 6, HA conjugated to polyamines such as polyethyleneimine (PEI) of 10 kDa molecular weight.

The presence of PEI helps the endosomal escape of the particles from endosomes, based on: “proton sponge effect” [26]. We synthesized the HA-PEI polymer 20 KDa, with a 40 mole percent PEI conjugated on the carboxylic group of the glucuronic acid monomer of the HA; we
also synthesized HA-PEG-modified polymer to use in the mixture with HA-PEI: such a mixture enables the control of particle size, avoids particle aggregation in the presence of the serum proteins, and facilitates evasion from immune system phagocytes [23]. As shown in Fig. 4, a thiol-modified HA polymer can be synthesized via a reduction EDS/NHS reaction with cysteamine: this polymer forms a polydispersed system if combined with the siRNA. Another modification made at the carboxylic group site of the glucuronic acid monomer, is the one that includes lipid chains of different lengths, such as C6, C8, and C10 (Fig.4). This library of HA modified polymers is intended to be used in a combinatorial approach that provides the positive charge for interaction with the negative charge of the siRNA, the PEG-modified (polyethylene glycol of PEG modified) polymer to help with the nanoparticle charge shielding, and a lipid modified polymer that might help the cell penetration of the nanoparticle. Alternatively, a thiolated polymer is also available in this library which can be used to perform appropriate conjugation reactions with the siRNA molecules, which increases the siRNA loading capacity [39].

### 2.2.2 Synthesis of modified siRNA duplexes

siRNA duplexes were synthesized at Alnylam Pharmaceuticals laboratory, on a controlled pore glass (CPG) solid support, with use of 2’ TBDMS protecting chemistry [22]. The process of manufacturing the single strands undergoes these steps: (i) synthesis, (ii) deprotection, (iii) purification, (iv) desalting, and (v) lyophilization. The single strands are duplexed and annealed by heating to 90°C, and then gradually cooled to room temperature. Schematic of siRNA manufacturing is shown in Figure 5.
Figure 5. Schematic that shows the process of siRNA manufacturing. To the left is shown the synthesis process of RNA single strand which goes through deprotection, coupling and capping, and oxidation or thiolation; to the right is shown flow chart of siRNA manufacturing process describing all steps involved. Courtesy of Alnylam Pharmaceuticals.

The TNF-α targeted sequence (anti-sense) and its sense strand were designed and screened at Alnylam using a proprietary bio-analytical algorithm. Single strands of the original siRNA are shown here. We plan to modify the sense strand and specify the terminal end accordingly.

(i) Sense strand: 5’ucuucuGucuAcuGAuAdTsdT3’

(ii) Antisense strand: 5’AAGUcAGuAGAcAGAAGAdTsdT3’ [40]

Note: Lower case letters represent O-Methyl modified building blocks at the 2’ position of sugar ring of the nucleotide, while upper case letters represent 2’TBDMS protected RNA building blocks at the sugar ring.
The sequences of the single strands shown here represent the native sequencing of strands of this siRNA, as presented in the research paper published by Alnylam’s scientists[40]. We will refer to as “the native siRNA”, to understand this siRNA. However, during the thesis work we synthesized a library of modified siRNA-s of the above shown one, at its sense strand. The siRNA delivery into the cells and tissues of interest is facilitated if both the delivery vehicle and the siRNA are modified appropriately, based on the characteristics of the target organ, tissue, or cells. For example, cholesterol molecules were conjugated at the 3’ end of the sense strand RNA (see (i) above), which was then duplexed with the anti-sense strand. Cholesterol conjugated siRNAs have shown a silencing efficacy in-vitro, based on previous studies [25]. We synthesized siRNA with a thiol linker on the 5’ end of the sense strand RNA shown in (i), to use with a conjugation reaction through a disulfide bond. As part of this library, we synthesized a fully P-S modified siRNA at its sense strand backbone. Several combinatorial approaches were applied to formulate the siRNA with the polymer, and a screening experiment was performed in order to select those modifications and formulations that show silencing efficacy on TNF-alpha gene, in in-vitro trial system.

2.2.3 Preparation and characterization of HA-based nanoparticle formulations

Figure 6 shows a general scheme of how the HA polymer is mixed with siRNA; this scheme applies for all experiments wherever a mixing formulating process is mentioned. The scheme is the same regardless of the siRNA modification, polymer modification, or polymer mixture.
Figure 6. Schematic that shows the process of mixing HA polymer with siRNA

2.2.3.1 Particle size analysis

A Malvern Instrument was used to characterize the size of nanoparticles that were manufactured as described above; the instrument uses Dynamic Light Scattering (DLS), measured by a detector positioned at an angle of 137° which enables capturing of a high intensity of the scattered light, at a temperature of 25° C. The sample is diluted in 1x DPBS buffer. Table 2 shows examples of the particle sizes and PDI values of different formulations. Relevance of the formulation is judged based on the particle size, PDI, and encapsulation efficiency.

Table 2. Nanoparticle characterization values for size, PDI, and zeta potential. Within the round parenthesis it is shown the polymer mixture ratio, while within the rectangular ones it is shown the polymer: siRNA ratio. The polymer modification is shown conventionally, while it is left to be understood that that particular modification is made on an Hyaluronic Acid polymer 20 KD.
2.2.3.2 Analysis of surface charge

The Malvern instrument was also used to measure the zeta potential of the nanoparticles. Measurements were made on particles diluted 2-fold with 1x DPBS. This method measures the particle surface potential, based on particle mobility in an electrophoretic field. The refractive index of the nanoparticle solution is 1.33. The viscosity of the dispersant is equal to that of the water 0.8872 cps.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Loaded siRNA--Polymer Mixture</th>
<th>Particle Size (nm ±S.D)</th>
<th>PDI (±SD)</th>
<th>Zeta Potential (mV ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-NU 01</td>
<td>[siRNA—(HA-PEI)] [1:44]</td>
<td>184±1.0</td>
<td>0.2±0.0</td>
<td>0.2±0.9</td>
</tr>
<tr>
<td>AL-NU 02</td>
<td>[siRNA—(HA-PEI:HA-PEG)] (1:1) [1:44]</td>
<td>117.3±0.4</td>
<td>1.2±0.0</td>
<td>-13.7±3</td>
</tr>
<tr>
<td>AL-NU 03</td>
<td>[siRNA—(HA-PEI:HA-PEG)] (2:1) [1:44]</td>
<td>134.4±0.7</td>
<td>0.1±0.0</td>
<td>-17.5±1.9</td>
</tr>
<tr>
<td>AL-NU 04</td>
<td>[siRNA—(HA-PEI:HA-PEG)] (3:1) [1:44]</td>
<td>146±0.5</td>
<td>0.1±0.0</td>
<td>-11.1±3.9</td>
</tr>
<tr>
<td>AL-NU 05</td>
<td>[Cholesterol siRNA—(HA-Decyl)] [1:40]</td>
<td>139.4±54.5</td>
<td>0.56±0.4</td>
<td>-16.3±5.2</td>
</tr>
<tr>
<td>AL-NU 06</td>
<td>[Cholesterol siRNA—(HA-Octyl)] [1:40]</td>
<td>189.7±72.0</td>
<td>0.5±0.3</td>
<td>-17.2±4.9</td>
</tr>
<tr>
<td>AL-NU 07</td>
<td>[Cholesterol siRNA—(HA-Hexyl)] [1:40]</td>
<td>269.2±63.3</td>
<td>0.4±0.1</td>
<td>-15.1±5.9</td>
</tr>
</tbody>
</table>
2.2.3.3 Transmission electron microscopy (TEM)

TEM analysis characterizes the particle by visualizing its morphology in an electron microscope. Samples are placed on coated copper grids, and then negatively stained with phosphor-tungstic dye at room temperature; particles are then observed on JEOL 110-X electronic microscope. The photomicrograph in Figure 7 shows a siRNA formulation with a mixture of HA-PEI/HA-PEG, in a 2:1 ratio. Particle size was about 100nm, and correlated with that measured on the Malvern Instrument for this formulation.

![TEM pictures on loaded HA-PEI particles with siRNA. Picture on the left shows nanoparticles of HA-PEI/HA-PEG mixture polymer at a 2:1 ratio, and siRNA at 1:10 ratio with the polymer; magnification of 3000 X. Picture on the right is a higher magnification view of the same nanoparticle to show a size of 100 nm (40-000 X). Note the aggregation which explains the values of PDI at 0.2.](image)

2.2.3.4 Encapsulation efficiency measured with use of the RiboGreen Assay

Ribogreen RNA reagent is a nucleic acid stain. Using fluorescein emission excitation wavelengths, this assay can detect RNA levels as low as 1ng/mL. The maximum excitation is 500 nm, and maximum emission is 525 nm. A standard curve is created with a set of known concentrations of RNA. Fluorescence measurements are made of the nanoparticle solution mixed with RiboGreen reagent: free siRNA binds to the RiboGreen reagent and fluorescence is positively correlated to the concentration of free siRNA. Then polyacrylic acid is used to disrupt
the nanoparticle and release the encapsulated siRNA, which allows us to obtain the total siRNA in the solution. Standard curves must have a correlation coefficient of > 0.99.

2.2.3.5 Measurement of endotoxin presence in the formulations

Lipopolysaccharides (also called endotoxins) up-regulate TNF-α target. We needed to formulate an endotoxin-free particle to avoid uncontrolled up-regulation of the target, and to prevent disturbances of mRNA levels during the silencing experiments. We tested the HA polymer and the siRNA for their endotoxin content making use of the Limulus Amebocyte Lysate (LAL) PTS cartridges method developed by Charles River Laboratories.

_The principles of the PTS method_ Bacterial endotoxins that are present in a sample activate a cascading series of serine proteases. The last step of the cascade is chromogenic, and the intensity of the color can be measured by a built-in spectrophotometer provided with a device called a PTS. The scheme of the enzymatic reaction is shown here:

![Enzymatic Reaction Diagram](image)

2.3 Results and Discussion

2.3.1 Preparation of nano-sized formulation

The technique utilized for making the nano-sized formulation yielded a unimodal size distribution with a narrow PDI value, every time the PEI modified HA polymer was used in the formulation, or as its part. The size of the formulation changes according to the components used for making the nanoparticle. It is noticed that HA lipid modified polymers used to formulate the
siRNA, generate polydispersed formulation and low encapsulation efficiency. However, when the HA-PEI is used in the formulation combined with other HA polymer components, the polydispersity index of the nanoparticle and siRNA encapsulation efficiency are improved significantly.

### 2.3.2 Discussion of the results for the formulation process

In Table 2 above was shown a summary of the results for the size, Poly Dispersity Index (PDI), and zeta potential of the formulations made in effort to encapsulate the siRNA. In Table 3 below, is shown a summary of the results for size, and PDI for the formulations made in effort to optimize the formulation in terms of size distribution, and siRNA encapsulation efficiency. It is clearly noticed that the presence of the HA-PEI is crucial for particle formation as well as for the siRNA encapsulation efficiency. This becomes more evident if the siRNA encapsulation efficiency is measured using the RiboGreen assay, as shown in Table 4.

**Table 3.** Nanoparticle optimization. Within the round parenthesis it is shown the polymer mixture ratio, while within the rectangular ones it is shown the polymer:siRNA ratio. The polymer modification is shown conventionally, while it is left to be understood that particular modification is made on a Hyaluronic Acid polymer 20 KD.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Loaded siRNA-Polymer Mixture</th>
<th>Particle size ±SD</th>
<th>PDI±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-NU 08</td>
<td>[(C6:PEI:PEG): siRNA Chol ] (3:2:1)[10:1]</td>
<td>76.0±6.9</td>
<td>0.19±0.03</td>
</tr>
<tr>
<td>AL-NU 09</td>
<td>[(C6:PEI:PEG): Chol siRNA] (5:2:1)[10:1]</td>
<td>76.4±7.9</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>AL-NU 10</td>
<td>[(C6:PEI:PEG): Chol siRNA] (6:1:0.5)[10:1]</td>
<td>107.9±21.4</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>AL-NU 11</td>
<td>[(C6:PEI:PEG): Chol siRNA] (1:3:1)[10:1]</td>
<td>70.1±1.1</td>
<td>0.16±0.0</td>
</tr>
<tr>
<td>AL-NU 12</td>
<td>[(C6:PEI:PEG): Chol siRNA] (2:3:1)[10:1]</td>
<td>66.6±1.9</td>
<td>0.19±0.0</td>
</tr>
<tr>
<td>AL-NU 13</td>
<td>[(C6:PEI:PEG): Chol siRNA] (2:5:1)[10:1]</td>
<td>87.8±3.1</td>
<td>0.212±0.0</td>
</tr>
</tbody>
</table>
Table 4. siRNA encapsulation efficiencies for the nanoparticles formed with HA-PEI and HA-Alkyl Polymers. The numbers, represent the ratio between the polymer components. The polymer modification is shown conventionally, while it is left to be understood that, that particular modification is made on an Hyaluronic Acid polymer 20 KD.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Free siRNA (mg/mL)</th>
<th>% Encapsulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-Decyl/siRNA</td>
<td>0.5</td>
<td>20.0</td>
</tr>
<tr>
<td>HA-Hexyl/siRNA</td>
<td>0.2</td>
<td>17.9</td>
</tr>
<tr>
<td>HA-Octyl/siRNA</td>
<td>0.5</td>
<td>15.6</td>
</tr>
<tr>
<td>HA-PEI/siRNA</td>
<td>0.00 (below the det. limit)</td>
<td>100.0</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 3:2:1</td>
<td>0.9</td>
<td>99.1</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 5:2:1</td>
<td>0.6</td>
<td>99.4</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 6:1:0.5</td>
<td>1.1</td>
<td>98.9</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 1:3:1</td>
<td>0.6</td>
<td>99.5</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:3:1</td>
<td>0.4</td>
<td>99.6</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:5:1</td>
<td>0.9</td>
<td>99.1</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:7:1</td>
<td>0.5</td>
<td>99.5</td>
</tr>
<tr>
<td>PEI:PEG 10:1 Chol</td>
<td>1.3</td>
<td>98.7</td>
</tr>
<tr>
<td>PEI:PEG 10:1 Unmodified</td>
<td>2.6</td>
<td>97.4</td>
</tr>
<tr>
<td>C6:PEI:PEG 1:1:1 Unmodified</td>
<td>1.1</td>
<td>98.9</td>
</tr>
</tbody>
</table>

In order to visualize the particle, we observed and took pictures of one of our formulations made with HA-PEI and HA-PEG polymer mixture. Figure 7 above showed the
TEM images, of this nanoparticle, which is made with unmodified anti TNF-alpha siRNA, and a mixture of HA-PEI with HA-PEG. This images and data from the DLS size distribution indicate that we are able to formulate spherical shaped particles, which most likely entrap the siRNA inside their core. Our proposed model for this particle is shown in Figure 8. If this is the case, during our work we have to encounter two problems related to the RNAi. First, this nanoparticle has to be taken up by macrophages; and second, the siRNA has to be released into the cytoplasm for the RNAi mechanism to occur. Data from the library of the formulations we made by combining siRNA with polymer mixture indicate that it is still possible to make a nano-sized particle we combine cholesterol modified siRNA with polymer mixture. More importantly, the introduction of the polymer that carries lipid moiety besides the PEI and PEG conjugated molecules, is likely to contribute into the nanoparticle formation through hydrophobic effect added up to the charge interaction between siRNA and PEI on the polymer. Our proposed model for this particle is shown in Figure 9.

**Figure 8.** Proposed model of the nanoparticle encapsulating the unmodified siRNA
2.4 Conclusions

The encapsulation of the siRNA in HA polymer can be reproducibly made by simple mixing method, which is facilitated by the solubility in water of both siRNA and HA polymer. In addition, the characterization of formulations demonstrated that the nanoparticles were between 80nm and 100nm in size, spherical and about -11 mV surface charge. Modifications applied to both siRNA and HA polymer helped to improve the drug loading and encapsulation efficiency. More importantly, these modifications are expected to contribute into the RNAi mechanism of action. The approaches shown here are likely to provide an exciting combinatorial solution with chemistry effort, to improve the delivery of siRNA into macrophages using a polymer vehicle that to start with, is known to have affinity for CD44 receptors expressed on macrophages.
CHAPTER 3

IN VITRO EVALUATIONS OF NANOPARTICLE UPTAKE AND GENE SILENCING IN MACROPHAGES

3.1 Introduction

Given the complexity of the immune system, it is not easy to study the crosstalk between immune cells. However, quite often in-vitro systems have been developed using macrophage cell lines which can be induced in function to the inflammation condition which is under investigation [41]. Macrophages derive from monocytes and are tissue-residing cells. These cells play a wide complicated role that encircles both non-specific and adaptive functions. In addition to pathogen engulfment and chemotaxis, macrophages are key players in the inflammatory stimulating process of other immune cells, because they present antigens to lymphocytes [42]. Murine macrophage cell lines like J774 or RAW 264.7 are extensively used in well-established model systems in cell biology and immunology. More importantly, these macrophages can easily be maintained and genetically manipulated. Due to these features, they are preferred models in eukaryotic immune-mechanics [42]. Important feature of macrophages is their ability to engulf foreign “particles” that happen to float around them. As the name indicates, (makros "large" + phagein "eat"), they are specialized in digesting the cellular debris, foreign substances, microbes, and cancer cells in a process called phagocytosis. Lam, et al., studied the mechanical characterization of J774 macrophages. In Figure 10 (a) a picture of resting macrophages is shown aside with an image of macrophages which have engulfed beads of 30 μm size (Figure 10 (b)) [42]. It is clearly shown how the typical wrinkles on their surface almost iron out at the time of engulfment. This typical feature is very often used in order to target macrophages with use of appropriate formulated particles that in turn deliver a drug of interest. Based on these findings,
we used a J774 A1 cell line to develop the siRNA delivery process in macrophages in order to silence TNF-α gene. Our rationale is that: if we deliver siRNA in macrophages and silence the TNF-α gene up-regulation with an impact in TNF-α cytokine excretion, we can potentially affect the release of the cytokine storm down the signaling stream. TNF-α is known to be the main mediator of inflammation.

Figure 10. In a) left, SEM micrographs of resting J774 macrophages, scale bar =10 μm are shown. In b) right, a composite of two SEM micrographs of J774 macrophages engulfing Fcγ-opsonized 30 μm (nominal diameter) beads. Whereas the macrophage at the top is still in the process of engulfment, the bottom cell has fully enclosed the target bead [42]

3.2. Cell Uptake Studies

3.2.1 Materials and methods
J774 A.1 macrophages from mouse ascites tissue were bought from ATCC. The cells were cultured in Dulbecco’s Modified Eagle’s Medium, Cat No. 30-2002. The medium was enriched with 10% Fetal Bovine Serum. Subcultures were prepared by scraping. In our practice, we had to transfect the cells with siRNA nanoparticle, so the use of trypsin to detach the cells was voided. For culturing 75 cm² plates Corning T075 (Cat#43641) were used. Cultivation ratio used was 1:6, and usually passages from 2 to 6 were used for the experiments.
3.2.2 Results and Discussion

The course of HA nanoparticle uptake by the cells was confirmed with use of confocal microscopy. A nanoparticle was formulated as shown in Chapter 2. The ratio Polymer: siRNA was preserved at 10:1 respectively. The HA-PEI was labeled with FITC dye (shown in green in the pictures), whereas the siRNA was labeled with ALEXA 647 (shown in red in the pictures). J774 A.1 cells (passage 4) were transfected with HA-PEI particles formulated with siRNA. A 48, 24, 6, 4, 1 hour transfection was established. At each designated time point cells were fixed with 4% paraformaldehyde for 10 min, rinsed with DPBS and stored in DPBS until analyzed. Hoescht nuclear stain 1:5,000 (in DPBS) was added in each well 15 to 30 min prior to the microscopy. Figure 11 a) shows images of uptake of free nanoparticles by cells, recorded at different time points. Figure 11 b), documents quantification of polymer and siRNA uptake (data extracted from the software output).
Fig 11. Quantitation of FITC Polymer and ALEXA 647-siRNA Uptake. The HA nanoparticle uptake proceeds from 4 to 24 hours and then plateaus out by 48 hours. The Polymer-siRNA ratio is maintained at the 10:1.

As the results suggest, it is noticed that as early as 4 hours after transfection, the cells start taking the nanoparticle up. The uptake increases progressively up to 24 hours, when the uptake plateaus out. At 48 hours there is no visual change as compared to 24 hours. Both polymer and siRNA seem to be co-localized within the cells starting at 4 hour time-point. Our understanding is that the uptake is endocytosis mediated, helped by the affinity CD44-HA. Although we can visually notice fairly well uptake of the nanoparticles by the J774 A.1 cells, we cannot conclude that the siRNA is released in the cytoplasm, in order for the RNAi to be mediated. More importantly these pictures do not show whether or not we are successful in silencing the gene of interest, TNF-α. Uptake is not necessarily translated into silencing efficacy. More studies will be carried out in molecular level, in order to assess the silencing effect that our nano-system might mediate into macrophages. Details and data will be shown here.

3.2.3 Conclusions

As expected, macrophages take up the nanoparticle successfully through what is most probably an endocytosis effect facilitated by the affinity of receptor-ligand relation between HA-PEI and CD 4 receptors expressed on macrophages. The RNAi mechanism is generally complicated; particularly it is our goal to dig dipper into the system we have created so far, and optimize it.

3.3. In Vitro Gene Silencing Studies

3.3.1. Materials and methods
Efficacy of mRNA silencing was assessed using the RT-qPCR kit from Invitrogen. The assay was developed according to the manufacturer’s protocol. Briefly, for the transfection we used the RNAiMAX Kit; Reverse Transcriptase (RT) Kit was utilized to synthesize the complementary DNA (cDNA) with use of reverse transcription starting from the total purified RNA from cells. The Dyno Beads Protocol was used for purifying RNA from the transfected cells, and cDNA was synthesized using a thermal cycle from Applied Biosystem. A TaqMan® TNF-α probe was used for the qPCR step followed by real time fluorescence reading on a Roche LiteCycler Reader. Changes in mRNA levels across multiple samples were determined by relative quantification, and the result expressed relative to levels of the GAPDH housekeeping gene, which is co-amplified along with the gene of interest. Calculations were based on a comparison with distinct cycle threshold (Ct) values at a constant level of fluorescence. The measured levels of mRNA in the cell were expressed as a number from 0 to 1 (equivalent to from 0 to 100%). All experiments were performed in duplicate.

3.3.2 Choosing positive and negative siRNA systems for future experiments

First, we developed the RT PCR protocol, with use of lipofectamine as the transfecting agent for 24 hours. Three siRNAs (1, 2, and 3), which are known to have efficacy in silencing TNF-α were screened as positive controls; a scrambled siRNA known to have no effect on the target, was screened as a negative control; a second scrambled siRNA, which targets the luciferase gene was another negative control. The siRNAs were taken from Alnylam’s inventory, based on the previous work from Alnylam’s scientists. The purpose of this experiment was to choose an anti-TNFα sequence that we could work with, and a negative control that we could use throughout our in-vitro experiments. The sequencing for siRNA1 and 2 are not shown here, but siRNA 1 has been published, so we show the sequence details throughout this thesis work [40].
Data were calibrated against mRNA levels in naïve untreated cells. A couple of negative control siRNAs (indicated as siRNA 4 and 5) were used for transfection as a reference point.

### 3.3.3 Creating and optimizing a nanoparticle system for transfection

Our goal is to create an efficacious delivery system of siRNA encapsulated in HA polymer that mediates RNAi for TNF-α silencing. After choosing the siRNA with positive and negative role from a developing experiment, we encapsulated the siRNA in a modified HA polymer nanoparticle that has been optimized continuously. Several approaches were used and are explained step by step on the results and discussion section below. We used naïve J774 A.1 macrophages and transfected them via forward transfection, then measured the TNF-α mRNA levels in the cells. We formulated a nanoparticle using a defined ratio in weight between the polymer and siRNA, as explained in the respective paragraphs. Cells were induced for 6 hours with 100ng/mL LPS, then transfected with the HA-PEI/siRNA nanoparticle for 24 hours (at 3 hours the transfecting media is replaced with plain DMEM medium). The concentration of the siRNA in the formulation is explained below. At the designated time point, total RNA was extracted and RT-qPCR was performed to quantify the levels of TNF-α mRNA in the cells. We used GAPDH as a housekeeping gene to calibrate the data.

### 3.3.4 Results and Discussions

We checked the silencing efficacy of the siRNA delivered by the Lipofectamine® cationic lipid-based transfection system. The results are shown in Figure 12. Based on the level of TNF-α mRNA measured from this developing assay, the negative control siRNA 5 is chosen as reference, since no effect on TNF-α was observed. Unlike siRNA5, scramble siRNA 4 showed upregulation relative to untreated cells, so we will not be using this scramble sequence for our
upcoming experiments. Also, based on these data we have chosen siRNA-3 as our active siRNA, which results in 59% TNF-α knockdown in J774 A.1 macrophages and siRNA-5 as the negative control, with no effect on levels of TNF-α mRNA. Throughout this text siRNA-3 is referred to as the non-modified or active siRNA, while siRNA-5 as the negative control or scramble.

![Silencing Assay Assay Development Using Lipofectamine](image)

**Figure 12.** Selection of a potent active anti TNF-α siRNA and a negative control siRNA. mRNA level scale from min 0 to max 1. The levels above 1 show gene up-regulation levels below 1 show down-regulation. 1 represents an active siRNA used previously for TNF-α silencing; 2 represents an active sequence found via a screening process at Alnylam Pharmaceuticals (sequencing not shown); 3 represents the active sequence developed at Alnylam, sequencing shown in here; 4 represents a scramble sequence used as a negative control found in previous publications; 5 represents a negative control siRNA which targets luciferase gene.

Moving forward, we encapsulated the selected siRNAs within HA-PEI polymer nanoparticles. Inflammatory responses of J774 A.1 cells were assessed after challenging them with LPS and measuring the level of mRNA TNF-α. We formulated a nanoparticle using a ratio 40:1 in weight between the polymer and siRNA: cells were induced for 6 hours with 100 ng/mL LPS, then at 6 hours they were transfected with the HA-PEI/siRNA nanoparticle for 24 hours
(the transfecting media was washed at 3 hours and replaced with DMEM plain medium until 24 hour time point). The concentration of the siRNA in the formulation was estimated to be 100 nM. At 24 hours RNA was extracted and RT-qPCR was performed to quantify the levels of TNF-α mRNA in the cells. Results are shown in Figure 13. As data indicates, TNF-α mRNA levels were elevated in stimulated cells compared to untreated cells as expected, due to the inflammatory response. However, no silencing was observed when cells were treated with HA-PEI/ siRNA for 24 hours and 100 nM siRNA, relative to control cells treated with HA-PEI/negative siRNA. Based on these results, we tested two hypotheses. **Hypothesis 1:** the nanoparticle gets entrapped in the endosome compartment, which stops the siRNA from being released in the cytoplasm. **Hypothesis 2:** siRNA release from the nanoparticle within the cell occurs as a function of time.

**Figure 13.** Silencing efficiency in LPS induced cells transfected with HA-PEI/siRNA nanoparticle. Minimum mRNA level is 0; maximum mRNA level is 1. The levels above 1 show gene up-regulation; levels below 1 show down-regulation.
We designed an experiment in which chloroquine was used to provide the endosome with buffering capacity for possible siRNA release [26]. The cells were transfected with HA-PEI/siRNA 40:1 weight ratio formulation for 100nM siRNA, and chloroquine was added for 50µM concentration in the well. Results are shown in Figure 14. This data indicates that it is likely that the nanoparticles get entrapped in the endosome. This is a well-known phenomenon from the past research studies, and a hurdle in the RNAi particularly and nanotechnology in general.

![Figure 14](image.png)

**Figure 14.** Silencing efficiency in induced cells transfected with HA-PEI/siRNA nanoparticle in the presence of 50µM chloroquine. Minimum mRNA level is 0; maximum mRNA level is 1. Levels > 1 show gene up-regulation; levels <1 show down-regulation. The levels of mRNA are decreased by 54% in the induced cells in the presence of the chloroquine, compared to the mRNA levels in cells transfected with the negative siRNA/HA-PEI complex in the presence of chloroquine. Addition of (HA-PEI/Neg siRNA-chloroquine triggers the up-regulation of mRNA as it is noticed by the comparison to the untreated cells.

An artistic image of endosomal entrapment (escape) is shown in Figure 15. [26].
Figure 15. Artistic representation of therapeutic internalization and endosomal entrapment followed by endosomal escape

Our understanding is that our delivery system needs further optimization in order to enable the siRNA release in the cytoplasm, after creating necessary conditions for endosomal escape. On the other hand, the 24 hours transfection may be insufficient time for siRNA release into the cell cytoplasm, which is necessary for initiation of the RNAi. We combined a time point experiment with a siRNA concentration screening based on polymer/siRNA ratios in the formulations. Cells were induced with 100 ng/mL LPS for 6 hours, then transfected with HA-PEI/siRNA nanoparticles. For the different concentrations of siRNA the transfection was stopped
at the designated time points and total RNA was extracted from cells, for TNF-α mRNA evaluation. Table 5 shows the formulation ratios between HA-PEI and siRNA, particle size, PDI. Zeta potential measured in a 20:1 dilution with DPBS is also shown.

**Table 5.** Formulation ratios, particle size (±SD), PDI (±SD), and Zeta Potential of nanoparticles used for the experiment.

<table>
<thead>
<tr>
<th>Weight Ratio Polymer: siRNA</th>
<th>2:1</th>
<th>4:1</th>
<th>7:1</th>
<th>10:1</th>
<th>10:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA conc. (nM)</td>
<td>540</td>
<td>269</td>
<td>154</td>
<td>108</td>
<td>54</td>
</tr>
<tr>
<td>Hydrodynamic Diameter ±SD (nm)</td>
<td>173.9±1.5</td>
<td>176.8±1.9</td>
<td>194.3±1.5</td>
<td>202.1±0.8</td>
<td>277.3±2.9</td>
</tr>
<tr>
<td>PDI±SD</td>
<td>0.1[43]±0.02</td>
<td>0.1±0.04</td>
<td>0.01±0.02</td>
<td>0.1±0.0</td>
<td>0.15±0.0</td>
</tr>
<tr>
<td>Zeta Potential (mV)</td>
<td>0.3</td>
<td>0.0</td>
<td>-0.0</td>
<td>0.0</td>
<td>-0.7</td>
</tr>
</tbody>
</table>

Results of the silencing are shown in Figures 16 and 17, and suggest that the most efficacious formulation was the 108nM siRNA: it achieved a 50% decrease in mRNA levels after 48 hours, followed by a 36% silencing at 72 hours and a 34% silencing at 96 hours. At 48 hours 20% silencing was observed for the 269 nM and 154 nM siRNA formulations, but the levels were not maintained beyond this time point. Approximately 50% silencing was obtained for the 540 nM formulation at 72 hours, but the result was not maintained at later times. A concentration this high is not practical for clinical use, anyways. Our data thus suggested that a 10:1 weight ratio HA-PEI: Polymer-siRNA should be maintained. We wanted to confirm this data by repeating same conditions: 100nM at 48 hours transfection, and 10:1 Polymer:siRNA ratio. The results were not consistent, indicating that we further need to optimize the delivery system for more efficient siRNA release into the cell cytoplasm.
Figure 16. Silencing efficiency of HA-PEI/siRNA formulations, expressed in mRNA level compared to GAPDH at 24 and 48 hours.
Chemically modified siRNAs, with the same sequence as the one of the active siRNA, were synthesized. We initially fully phosphorothioated (P=S) the backbone on the sense strand, then combined it with equivalent amount of the antisense strand to make a duplex siRNA. Backbone modifications may cause loss in binding affinity, but do not affect the silencing ability of siRNAs, may cause toxicity, but increase the resistance to nucleases[22]. A cholesterol molecule was conjugated at the 3’ end of the sense strand then both strands were combined to form a duplex. Cholesterol-modified siRNA reportedly does display activity in silencing the apolipoprotein B (ApoB) gene [25]. Then, we formulated the modified siRNA-s with HA-PEI polymer for targeted delivery in macrophages. Results suggest that: (i) Cholesterol-conjugated
siRNA results in 25-30% silencing when transfected with lipofectamine, or when no transfecting agent is used. When the siRNA is formulated with HA-PEI no silencing is observed. The result Figure 18, suggests that HA-PEI polymer formulated with the siRNA does not release the siRNA within the cell. (ii) For the P=S-modified siRNA, the result matches reports in the literature. The P-S modification on the backbone triggers immune response [22]. TNF-α up-regulation is observed with use of lipofectamine transfection and the HA-PEI formulation. Up-regulation was greater for the HA-PEI nanoparticle with broad variability. The most interesting result, that gets our attention at this point, is the fact that for the cholesterol modified siRNA, the nanoparticle encapsulation affects the silencing efficacy. This indicates that we still need to optimize the nanoparticle system, keeping the HA polymer: siRNA weight ratio constant at 10:1.

**Figure 18.** Results of silencing, with modified siRNA (100 nM, 48 hours transfection). Eight results are shown. Each result is represented by two bars. Blue bar represents the modification or formulation of interest. Red bar represents the respective negative control. Result 1 represents the original siRNA silencing effect using lipofectamine for transfection; 2 represents the Ha-PEI polymer formulated with siRNA; 3 represents cholesterol modified siRNA effect using lipofectamine to transfect; 4 represents the cholesterol modified siRNA with no transfecting agent present; 5 represents chol-siRNA formulated with HA-PEI; 6 represents fully P-S modified siRNA transfected with lipofectamine; 7 represents fully P-S modified siRNA formulated with HA-PEI polymer;
Table 6. Size and PDI of nanoparticles loaded with siRNA after dialysis. Standard deviation is shown side by side. The convention C6:PEI:PEG stands for HA-C6: HA-PEI:HA-PEG mixture of the same weight concentration followed by the numbers representing the mixture ratio in volume. “Chol” and “unmodified” stand for cholesterol modified siRNA and unmodified siRNA respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size ±SD (nm)</th>
<th>PDI ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6:PEI:PEG Chol 3:2:1</td>
<td>76.02±6.9</td>
<td>0.2±0.0</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 5:2:1</td>
<td>76.4±7.9</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 6:1:0.5</td>
<td>107.9±21.4</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 1:3:1</td>
<td>70.1±1.1</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:3:1</td>
<td>66.6±1.9</td>
<td>0.2±0.0</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:5:1</td>
<td>87.8±3.0</td>
<td>0.2±0.0</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:7:1</td>
<td>85.4±3.7</td>
<td>0.2±0.0</td>
</tr>
<tr>
<td>PEI:PEG 10:1 Chol</td>
<td>96.3±0.8</td>
<td>0.2±0.0</td>
</tr>
<tr>
<td>PEI:PEG 10:1 Unmodified</td>
<td>176.1±4.9</td>
<td>0.2±0.0</td>
</tr>
<tr>
<td>C6:PEI:PEG 1:1:1 Unmodified</td>
<td>133.4±5.4</td>
<td>0.2±0.0</td>
</tr>
</tbody>
</table>

In support to specific aim 1 and specific aim 2 of this project, an important goal to achieve was the preparation of a HA nanoparticle loaded with anti-TNF-α siRNA that would not only have a silencing effect of the TNF-α gene in-vitro, but more importantly, have it at an earlier time point than 48 and 72 hours after transfection. We made various formulations were made loading the cholesterol modified siRNA and the unmodified siRNA at different mixture ratios not screened before. Briefly, the nanoparticles were characterized for size and PDI using DLS, and encapsulation efficiency using RiboGreen assay. The nanoparticles were dialyzed through 20 KDa cutoff membrane using 1xPBS overnight to remove free siRNA and the size and PDI were re-evaluated. Cells were transfected in regular medium containing 10%FBS and 5% Glutamate. The transfecting medium was removed at 4 hours and cells were cultured in plain DMEM medium without feeding factors for the rest of the time. This approach avoids excessive
cell growth from one time point to another and makes the conditions as similar as possible across the experiment. Cells were processed at the required time in the order:

\[ \text{RNA total extraction} \rightarrow \text{Combined DNA synthesis by reverse transcription} \rightarrow \text{Amplification of genes of interest using specific probes for detection on the Lite Cycler} \]

Beta-Actin instead of GAPDH was used as housekeeping gene for data normalization. There are indications that GAPDH, might not be the perfect choice as a home keeping gene when it comes to LPS induction in cells. Calculations were made using negative control cells transfected with scrambled siRNA. Other controls such as naïve cells were also used to check across the data. Later times than 72 hours were not evaluated as we had particular interest at 24 and 48 hour time point results. In the tables 7, and 8, are shown data for size, charge and siRNA encapsulation efficiency for the screened nanoparticles.
Table 7. Size and PDI of nanoparticles loaded with siRNA after dialysis. Standard deviation is shown side by side. The convention C6:PEI:PEG stands for HA-C6: HA-PEI:HA-PEG mixture of the same weight concentration followed by the numbers representing the mixture ratio in volume. “Chol” and “unmodified” stand for cholesterol modified siRNA and unmodified siRNA respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size ± SD (nm)</th>
<th>PDI ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6:PEI:PEG Chol 3:2:1</td>
<td>76.0±6.9</td>
<td>0.2±0.03</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 5:2:1</td>
<td>76.4±7.9</td>
<td>0.3±0.01</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 6:1:0.5</td>
<td>107.9±21.4</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 1:3:1</td>
<td>70.8±1.1</td>
<td>0.2±0.01</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:3:1</td>
<td>66.6±1.9</td>
<td>0.2±0.02</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:5:1</td>
<td>87.8±3.1</td>
<td>0.2±0.01</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:7:1</td>
<td>85.4±3.7</td>
<td>0.3±0.03</td>
</tr>
<tr>
<td>PEI:PEG 10:1 Chol</td>
<td>96.3±0.8</td>
<td>0.2±0.02</td>
</tr>
<tr>
<td>PEI:PEG 10:1 Unmodified</td>
<td>176.1±4.9</td>
<td>0.2±0.03</td>
</tr>
<tr>
<td>C6:PEI:PEG 1:1:1 Unmodified</td>
<td>133.4±5.4</td>
<td>0.2±0.05</td>
</tr>
</tbody>
</table>

Table 8. Encapsulation efficiency measured by RiboGreen assay, after dialysis is shown. RFU represents relative fluorescence units; PAA represents Poly Acrylic Acid used for nanoparticle disruption; TRIT represents triton used for disruption hydrophobic interaction between cholesterol on siRNA and hexyl group on the HA polymer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RFU NO PAA</th>
<th>RFU + PAA</th>
<th>RFU - PAA + TRIT</th>
<th>Free siRNA %</th>
<th>Encapsulated %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLK</td>
<td>0.7</td>
<td>8.3</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 3:2:1</td>
<td>1.1</td>
<td>60.2</td>
<td>93.1</td>
<td>0.9</td>
<td>99.1</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 5:2:1</td>
<td>1.1</td>
<td>77.9</td>
<td>116.3</td>
<td>0.6</td>
<td>99.4</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 6:1:0.5</td>
<td>1.2</td>
<td>52.8</td>
<td>85.4</td>
<td>1.1</td>
<td>98.9</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 1:3:1</td>
<td>1.1</td>
<td>93.4</td>
<td>115.3</td>
<td>0.6</td>
<td>99.5</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:3:1</td>
<td>1.1</td>
<td>104.3</td>
<td>128.1</td>
<td>0.4</td>
<td>99.6</td>
</tr>
<tr>
<td>C6:PEI:PEG Chol 2:5:1</td>
<td>0.9</td>
<td>36.9</td>
<td>63.7</td>
<td>0.9</td>
<td>99.1</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------------------------</td>
<td>------------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>33.6</td>
<td>21.1</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>59.1</td>
<td>40.6</td>
<td>20.9</td>
<td>30.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>67.8</td>
<td>1.3</td>
<td>2.56</td>
<td>32.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>98.7</td>
<td>97.4</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99.5</td>
<td></td>
<td></td>
<td>98.9</td>
<td></td>
</tr>
</tbody>
</table>

The rationale behind these formulations is that the introduction of a lipid component through a modified HA polymer with a lipid chain facilitates the siRNA release and nanoparticle endosomal escape. In the past a similar rationale was applied to formulate lipid nanoparticles [44]. Another expected benefit from this approach might be that a decrease in HA-PEI component in the formulation might result in the decrease of toxicity that the PEI moiety is known to trigger in various kinds of cells. More importantly, the macrophages we use for the *in-vitro* work are more sensitive to these kinds of stimuli. In an effort to attain earlier silencing effect of TNF-α, we formulated and screened these nanoparticles. In Figures 18, 19, 20, 21 we show figures of the results for the TNF-α silencing.

**Figure 19.** TNF-α silencing effect of nanoparticles made with HA-C6, HA-PEI and HA-PEG at 3:2:1 ratio between them. Cholesterol modified siRNA concentration 100 nM.
Figure 20. TNF-α silencing effect of nanoparticles made with HA-C6, HA-PEI and HA-PEG at 5:2:1 ratio between them. Cholesterol modified siRNA concentration 100 nM.

Figure 21. TNF-α silencing effect of nanoparticles made with HA-PEI and HA-PEG at 10:1 ratio between them. Cholesterol modified siRNA concentration 100 nM.
Figure 22. TNF-α silencing effect of nanoparticles made with HA-PEI and HA-PEG at 10:1 ratio between them. Unmodified siRNA concentration 100 nM

The data indicates that the presence of the lipid moiety in the formulation (i.e Figure 19), generates a fast and more transient silencing effect, which decreases with time. Whereas, for the formulation with more lipid moiety in the formulation the silencing (i.e. fig 20), the result is fast, and longer lasting. However, if we compare fig 21 with figure 20 we conclude that the cholesterol modified siRNA is active, but the presence of the lipid moiety in the formulation generates a more acceptable result if we consider that the TNF-α silencing should be transient given its importance in the host immune system. To confirm our results from previous experiments, in figure 22 we notice that the native siRNA formulated in HA-PEI/ HA-PEG mixture generates a delayed silencing beyond our time point of interest.

Based on these results we propose a model for the nanoparticle formulation that includes the lipid moiety where the intact forces are: the charge interaction between siRNA-PEI, and the hydrophobic effect of the cholesterol on the siRNA with the lipid on the polymer mixture. We strongly believe that a system like this promotes the nanoparticle endosomal escape, as well as the siRNA release in the cytoplasm. Figure 23 shows the artistic representation of this model.
3.3.5 Conclusions

The *in-vitro* work led to continuous optimization of the delivery system, through assessment of gene silencing, and inflammatory response. We have prepared hyaluronic acid (HA)-based CD44 targeted nanoparticle delivery systems that can efficiently encapsulate the siRNA duplexes; as indicated by the data it is likely that the system promotes RNAi in activated macrophages. However, the *in-vitro* results are just showing that we have achieved the goal of building a delivery system for siRNA delivery in macrophages. The complexity of the *in vivo* system is likely to raise interesting questions, and lead to further optimization.

3.4 Preliminary evaluations of anti-inflammatory response

3.4.1 Materials and methods

In support of Specific Aim 3, *in-vitro* we developed a mouse cytokine panel immunoassay based on manufacturer’s instructions. The general term “cytokine” is used for the group of proteins and peptides that modulate the functional activities of cells and tissues through their role...
as regulators under either normal or pathological conditions [45]. Cytokine and chemokine research plays an important role in achieving a deeper understanding of sepsis syndrome, especially under the theory that explains sepsis as a condition caused by a “cytokine storm” during inflammation process [46]. Briefly, the principle of this assay is based on the Luminex xMAP technology, which uses proprietary techniques to color-code microspheres with two fluorescent dyes internally. Using precise concentrations of these dyes, 100 bead sets can be created, each of which is distinguishable by a specific capture antibody. After the analyte of interest is incubated with the marked bead from the sample, a biotinylated detection antibody is added into the mixture. Streptavidin-PE conjugate is used as a reporter molecule, which is added in the reaction based on the protocol. Finally, the microspheres are allowed to pass rapidly through a laser which excites the internal dye and marks the microsphere set. A second laser excites the fluorescent dye on the reporter molecule. This assay offers the capability of adding multiple conjugated beads to each sample, which results in the ability to obtain multiple results from each sample.

The panel of 25 cytokines that were measured included: G-CSF, GM-CSF, INF-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-17, IP-10, KC, MCP, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α. The J774A1 macrophage cell line was used for the in-vitro assay in order to get preliminary information on macrophage lineage response to escalating LPS induction. Briefly, using a 96 well flat bottom clear plate, 50000 cells were plated per well in duplicate and allowed to attach for 6 hours. DMEM medium completed with 10%FBS was used for the 6 hours of attachment, then replaced with plain DMEM medium in order to keep cell growth to minimum rate for consistence throughout the time points. LPS induction was then applied at the indicated time. When the time was completed, the supernatant was removed,
transferred into a different 96 well plate and stored at -70°C until the experiment was completed. Then, the cytokine assay was performed following the manufacturer’s instructions. This simplified in-vitro model might not represent the in vivo response, however, it may help in identifying biomarkers with particular interest to use with the future in vivo experiments. In table 9 below the LPS concentration and time points are summarized.

Table 9. LPS concentrations and time points applied for the in vitro cytokine assay in J774A.1 macrophage lineage

<table>
<thead>
<tr>
<th>LPS concentration (ng/mL)</th>
<th>10</th>
<th>100</th>
<th>500</th>
<th>750</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time Point (hours)</td>
<td>6, 16 and 24</td>
<td>6, 16, and 24</td>
<td>6, 16, and 24</td>
<td>6, 16, and 24</td>
<td>6, 16, and 24</td>
</tr>
</tbody>
</table>

3.4.2. Results and Discussions

Figure 24 displays the plotted cytokine levels as measured by the software in ng/mL.
Figure 24. Released cytokine levels in the supernatant on plated *J774 A1* lineage cells induced with LPS at 6 hours, 16 hours, 24 hours

It is important to mention that the standard curves and the levels of cytokines in the standard solutions provided with the kit were all within the acceptance ranges, as required by the criteria for test suitability. The panel of cytokines that were measured is: G-CSF, GM-CSF, INF-γ, IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, IP-10, KC, MCP, MIP-1a, MIP-1b, MIP-2, RANTES, TNF-α.

The cytokines and chemokines that are plotted were the ones which resulted at detectable levels by the assay. These cytokines and chemokines: INF-γ, IL-2, 4, 5, 7, 9, 12, 13, 17, MCP, MIP-1, resulted in levels below the limit of detection of the assay, so are not displayed. A LPS dose response is observed for GM-CSF, IL-1a, IL-1b, IL-6, IP-10, MIP-2, RANTES, TNF-α. The cytokines and chemokines that are plotted were the ones which resulted at detectable levels by the assay. These cytokines and chemokines: INF-γ, IL-2, 4, 5, 7, 9, 12, 13, 17, MCP, MIP-1, resulted
in levels below the limit of detection of the assay, so are not displayed. A LPS dose response is observed for GM-CSF, IL-1a, IL-1b, IL-6, IP-10, MIP-2, RANTES, TNF-α. It is important to mention that decreasing levels of TNF-α are observed beyond a dose of 750 ng/mL which could be explained with macrophage desensitization. Also, progressively increasing levels of IL-6 were measured from 6 to 16 to 24 hours, along with a dose response. Interestingly, MIP-2 production undergoes upregulation from 6 to 16 hours but plateaus at 24 hours; however, a dose response is clearly achieved. Granulocyte-colony-stimulating-factor (GM-CSF) production was upregulated dose-dependently from 6 to 16 hours, but downregulation was observed after 16 hours and at 24 hours, its levels were back to the ones measured at the 6 hour time point.

Table 1 in Chapter 1, shows a short description of their role in inflammation.

3.4.3 Conclusions

Macrophages are sensitive to LPS and respond with an excessive cytokine excretion in the environment they exist [47]. This in vitro system contains only one macrophage lineage however, these findings, yet correlate with findings from previous studies for the in vitro stimulation of macrophages, as well as are consistent with the reported facts from in vivo studies on cytokine profile during the course inflammation. For this thesis work, the information is helpful given the fact that macrophages is the lineage that is being targeted using the HA polymer nanoparticles loaded with siRNA.
CHAPTER 4

IN VIVO MODEL AND STUDIES OF SILENCING EFFICACY

4.1 Introduction

Animal models of sepsis are based on use of three types of agents to trigger the disease: (i) administration of a toxin, like lipopolysaccharide (LPS) (ii) administration of a pathogen, like a gram negative bacteria (iii) alteration of the animal’s endogenous protective barrier, as by allowing bacterial translocation [48].

LPS- induced sepsis in animals, leads to rapid increases and high peak levels of pro-inflammatory cytokines compared to what has been reported for humans. However, when the amount of LPS administered is titrated to mimic the early stages of human disease, the model is particularly useful for studying systemic and renal responses during the early phases of sepsis [32].

The Cecal Ligation and Puncture (CLP) model is a polymicrobial model that leads to bacteremia followed by sepsis. Although not standardized, this model offers possibilities for studying the severity of the response by adjusting the length of the ligated cecum and the number of punctures. Similar to the CLP model is the colon ascendant stent peritonitis CASP model, wherein a stent is inserted to create a septic focus. Both methods are associated with a large variability from subject to subject, which often times underestimated [4]. In Figure 25 is shown an image of the CLP and CASP model.
Bacterial infusion or instillation models induce sepsis by introducing single pathogens in a controlled manner. Although the CLP and instillation models each have their own advantages and disadvantages, the instillation model offers greater reproducibility, and has been more readily transferred into larger animals, especially for the study of systemic or organ-specific hemodynamics [3, 4, 32].

Regardless of the model, there is a gap between animal models of sepsis and the human disease: the animal models lack use of intensive care facilities, raising the question of whether or not primate models are adequate surrogates for human sepsis.

We chose to create a simple LPS model for sepsis, by injecting LPS in the peritoneal cavity. A model like this facilitates the proof of siRNA action on silencing the TNF-α, by measuring the mRNA level in the peritoneal cells [48]. Besides the simplicity, using the LPS model we expect more consistency throughout the data given that, the immune system is diverse from subject to subject. However, in order to shrink the gap between the animal models and
human models, we paired the TNF-α observation with observation of a panel of cytokines and indicated the ones that most likely cross the TNF-α pathway.

4.2 Materials and methods

4.2.1. In vivo animal model development and materials

In vivo animal model

In support to our specific aims, an important step was to investigate the behavior of HA polymer nanoparticles in in vivo systems. Male C57Bl/6 mice, also called “C56 black 6” or simply “black 6”, is characterized by the strain stability, and is easily breeder. Second after the human one, its genome was fully sequenced in 2005. The most common area where this strain is widely used is the physiological or pathological models for in vivo experiments. While this type on mice is often used in immunology, during this thesis work we used it for creating a mouse model of sepsis. Using of LPS in mice is easy form the practical standpoint, but as it can be harsh if it is not applied carefully. Having said this, during this thesis work, we have extensively used an experimental set-up of local inflammation in the peritoneal cavity in mice. In order to study behavior of immune cells during inflammation and sepsis, using thioglycollate medium to recruit macrophages in the peritoneum cavity has been successfully used in the past [48]. We applied this approach to study the silencing efficacy of the HA polymer based nanoparticles that encapsulate the anti- TNF-α siRNA. Thioglycollate at 3% was injected via intraperitoneal route, at a reasonably large amount of 1mL per mouse. The experiment was designed in seven groups of animals, with 4 mice per group (n=4). The groups respectively represented: 1) LPS induced mice treated with scramble siRNA formulation, 2) LPS induced mice, 3) LPS induced mice treated with plain HA polymer, 4), 5), 6) LPS induced mice respectively treated with the test articles of FORM 1, FORM 2, and FORM 3, and 7) untreated group of mice as a control.
Materials

Listed here are the materials used and the purpose: a) Thioglycolate (TG) was made at 3% in 0.9% sodium chloride; b) LPS was made at 0.01 mg/mL in saline for 0.5 mg/kg dosing; c) test articles were supplied at 0.1 mg/mL siRNA (Alnylam) formulated in 1X PBS (Ca $^{2+}$, Mg$^{2+}$)-free with HA polymer (Northeastern University); c) **Form 1 (Chol)** is a formulation of TNF-$\alpha$ cholesterol modified siRNA in 1 mg/mL polymer mixture of: PEI-modified HA (2-5 moles %), PEG- modified HA (2-5 moles %), and hexyl modified HA (28-32 mole%) at volume ratios 2:1:5 respectively; **Form 2 (Chol)** is a formulation of TNF-$\alpha$ cholesterol modified siRNA in 1 mg/mL HA polymer mixture as explained in **Form 1**, but at volume ratios 2:1:3 respectively;  **Form 3** (active siRNA) is a formulation of **TNF-$\alpha$ siRNA** in 1 mg/mL HA polymer mixture like the one in **Form 2** at the same volume ratios 2:1:3 respectively; The siRNA dose is calculated for the final formulated nanoparticle; d) Unloaded Polymer is a mixture of 1mg/mL concentrated HA polymer as described for **Form 2** (chol) and **Form 3** (active siRNA) with no siRNA loading. All solutions were made in 1xPBS (Ca $^{2+}$, Mg$^{2+}$)-free. No dilution was required.

Below are shown the single strand sequences for unmodified and cholesterol modified duplexes of the anti- TNF-$\alpha$ siRNA.

**Duplex 1**

**Unmodified Sense strand:** 5’[ucuucuGucuAcuGAAcuuddTsdT]3’

**Unmodified Anti-sense Strand:** 5’[AAGUUcAGuAGAeAGAAGAdTsdT]3’

**Duplex 2**

**Unmodified Sense strand:** 5’[ucuucuGucuAcuGAAcuuddTsdT] 3’Chol
4.2.2. Experimental procedure

The method of administration was intra-peritoneal dosing in the peritoneal cavity for all groups. Macrophages were recruited in the peritoneal cavity using 3% TG medium in saline. The TG was not aged, but the animals were left with it for 72 hours before further treatment was started. The purpose for this amount of time was to allow macrophages migration to the peritoneum. Neither alteration was observed in animals’ health because of the TG, nor weight loss. From the point the animals were injected with siRNA nanoparticle until LPS injection, no health alterations were observed. After LPS injection, on the day of necropsy 2-2.5 gr weight loss was observed in all LPS-injected animals, while the control group did not exhibit any weight loss. None of the animals died during the experiment. It is known that the LPS effect on the TNF target gene is transient. We do not know what the peak time is for the TNF-α level in mice; therefore, we pre-treated the animals with siRNA nanoparticles before injecting LPS in the peritoneal cavity. The nanoparticles were prepared as described in the proposal defense. Based on the findings from the in vitro experiment we made a formulation of a mixture of HA polymers at 1 mg/mL concentration with siRNA for 1 mg/kg dosing. The experiment design is shown in the summarized table 10 below, along with dosing of each test article.
Table 10. Table that shows sequence of events during the silencing efficacy evaluation experiment

<table>
<thead>
<tr>
<th>Gr#</th>
<th>Test Article</th>
<th>Test Article</th>
<th>Test Article</th>
<th>Dose (mg/kg)</th>
<th>Conc (mg/mL)</th>
<th>Dose Volume (mL/kg)</th>
<th># of Animals per group</th>
<th>Route and Regimen</th>
<th>Day of Necro</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9% NaCl</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Scramble control</td>
<td>FORM 1 (Chol)</td>
<td>0.5mg/kg LPS</td>
<td>1.0</td>
<td>0.1</td>
<td>10</td>
<td>4</td>
<td>IP</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>3% Thioglycolate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>FORM 2 (Chol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>FORM 3 (active siRNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Unloaded Polymer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.9% NaCl (Saline)</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

At the day of necropsy, mice were anesthetized with isoflurane and blood was collected from ret orbital vein; then mice were euthanized and peritoneal cells were collected. The peritoneal cells were purified to obtain pure macrophages. After 30 min- 1 hour at room temperature, the blood was spun out to take the serum, then serum was stored at -20°C until further measurement was available.

4.2.3. Methods

The encapsulation efficiency was measured after final dialysis through 20 kDa cut-off membranes. The particles were characterized for size and PDI and the results are shown in the Figure 26 (top to bottom on the legend).
### Results

<table>
<thead>
<tr>
<th>Z-Average (d.nm):</th>
<th>78.11</th>
<th>116.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1:</td>
<td>98.63</td>
<td>104.7</td>
</tr>
<tr>
<td>% Intensity</td>
<td>100.0</td>
<td>96.8</td>
</tr>
<tr>
<td>Width (d.nm):</td>
<td>38.81</td>
<td>47.47</td>
</tr>
<tr>
<td>Pdi:</td>
<td>0.248</td>
<td>0.252</td>
</tr>
<tr>
<td>Peak 2:</td>
<td>0.000</td>
<td>54.18</td>
</tr>
<tr>
<td>% Volume</td>
<td>0.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.0</td>
</tr>
<tr>
<td>Width (d.nm):</td>
<td>0.0</td>
<td>648.6</td>
</tr>
<tr>
<td>Intercept:</td>
<td>0.930</td>
<td>0.939</td>
</tr>
<tr>
<td>Peak 3:</td>
<td>0.000</td>
<td>0.0</td>
</tr>
<tr>
<td>% Volume</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Result quality:</td>
<td>Good</td>
<td>Good</td>
</tr>
</tbody>
</table>

#### Size Distribution by Intensity

![Size Distribution by Intensity](image1.png)

#### Size Distribution by Volume

![Size Distribution by Volume](image2.png)
Figure 26. The size distribution of the nanoparticles before injection into animals. **From top to Bottom:**  
**FORM 1** 5:2:1 HA-C6:HA-PEI:HA-PEG/ Chol siRNA; Size: 98.63 nm, PDI: 0.248  
**FORM 2** 3:2:1 HA-C6: HA-PEI:HA-PEG/ Chol siRNA; Size: 104.7 nm, PDI: 0.252  
**FORM 3** 3:2:1 HA-C6: HA-PEI: HA-PEG/Unmodified siRNA; Size: 191.6 nm, PDI: 0.285  

The encapsulation efficiency was measured using the RiboGreen assay from Invitrogen according to the manufacturer’s instructions. Polyacrylic acid (PAA) at 2% in deionized H₂O was used to disrupt the particle and relative fluorescence units were measured. The results are shown in Table 11.
Table 11. Encapsulation efficiency of nanoparticles measured by Ribogreen assay. Before poly(acrylic acid (PAA) and after PAA addition represent, respectively, the fluorescence measurement before and after disrupting the nanoparticles with a counterion poly(acrylic acid).

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Scramble</th>
<th>FORM 1</th>
<th>FORM 2</th>
<th>FORM 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before PAA</td>
<td>1.1</td>
<td>4.9</td>
<td>2.4</td>
<td>1.5</td>
<td>1.01</td>
</tr>
<tr>
<td>After PAA</td>
<td>3.8</td>
<td>901.3</td>
<td>98.1</td>
<td>80.2</td>
<td>76.7</td>
</tr>
<tr>
<td>Encapsulation %</td>
<td></td>
<td>99.6</td>
<td>98.6</td>
<td>99.5</td>
<td>98.6</td>
</tr>
</tbody>
</table>

The calculations are made according to the standard curve built with known concentration solutions of siRNA Figure 27.

Figure 27. a) Standard curve that determines the free siRNA concentration in the set of test samples. Top: No Acrylyc acid is used. Bottom: Standard curve used for measuring the total siRNA freed from particles treated with 2% Polyacrylic acid in water.
After obtaining the peritoneal lavage, we purified the cells on the same day, by means of a protocol provided by Miltenyi Biotec that uses magnetic beads. Briefly, this protocol applies manual separation of leucocytes on labeled microbeads placed in a magnetic field. The columns contain a hydrophilic optimized matrix, which allows the creation of a high-magnetic field gradient. The leucocytes get retained on the matrix via magnetic interaction, while the other cell types wash through the column into a tube. Then, the macrophages are washed out with buffer provided with the kit when the tube is removed from the magnetic field. The protocol is validated to generate a pool of macrophages with greater than 80% purity. The cells were lysed using lysis buffer and stored at -70°C until further analysis. To check the quality of separation, we stained the cells with CD11 antibodies and performed FACS analysis. We evaluated this as an important step for assessing the purity of the cells that would undergo qPCR analysis to determine the TNF-α mRNA levels. For practical reasons, we pooled cells from the 28 animals of the whole experiment and performed the FACS analysis in duplicated pooled sample. The results from FACS analysis are shown in figure 28.

**Figure 28.** Results from FACS analysis of a pool sample of macrophage cells separated by means of magnetic beads MACS protocol
The obtained result of 85% pure macrophages aligns with the manufacturer’s instructions claim that the protocol assures at least 85% pure macrophages. In order to measure TNF-α mRNA level in peritoneal macrophages, we applied the same methods as the ones described in Chapter 3, section 3.3.1. Briefly, we extracted total RNA from cells using the Dynobeads protocol; then the qPCR was run to estimate the TNF-α mRNA level. The results were normalized towards β-actin.

4.3 In vivo results of TNF-α gene silencing

The results for the siRNA treated groups were normalized against the results from the group treated with the scrambled sequence. The scrambled treated group was normalized against the results from the group treated with LPS only. However, in order to check on the LPS effect on TNF-α, we checked its levels in the cells harvested from the untreated animals. We find it necessary to mention that, while comparing the group of mice that was only treated with LPS versus the untreated animals, we observed that the levels of TNF-α did not exceed 3 fold increase.

Figure 29. TNF-α level in the LPS treated group of mice versus the untreated group.
We explain this with the fact that the cells were harvested from animals after 24 hours, which may be a longer time than the TNF-α peak in LPS treated macrophages. Previous studies have shown much earlier times for the TNF-α peak expression [49]. For this experiment, we dosed the animals with 0.5 mg/kg LPS once and performed our ex-vivo measurements in cells, serum and tissues at one time point of 24 hours. We had an outlier present in the LPS treated group, which cannot be explained scientifically. The outlier presence could be explained by the fact that mice are resistant to LPS, as compared to other species including humans. More importantly, we injected a low LPS dose. As this is the first in vivo experiment, we are not highly confident in how the LPS stimulation behaves, which might prompt for careful observation during the future experiments.

In Figure 30, we show the results of the control groups, scramble siRNA treated and plain HA polymer treated groups, versus the LPS treated group. Corresponding table is also shown.

Table below that corresponds to Figure 30 below.

<table>
<thead>
<tr>
<th></th>
<th>TNF-α mRNA/ β-Actin</th>
<th>St Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LPS Only</strong></td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Scramble</strong></td>
<td>1.1</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Polymer</strong></td>
<td>1.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Figure 30. TNF-α level in the control groups compared with the group of mice treated with LPS. Results in % (1 stands for 100%). ANOVA shows no difference between groups.

The results indicate some variability within the animals of the same group, which is represented by rather high error bars. However, ANOVA did not show significant difference between LPS only treated group on one hand, and plain HA polymer +LPS treated and scramble siRNA +LPS treated on the other hand (p-value= 0.33). First, this result enables us to use the scramble siRNA treated group as our negative control group; and second we can infer that the polymer by itself does not show any stimulating effect on our target, as well as the scramble siRNA we have chosen is appropriate to use with control groups of mice. However, it is likely that the error bars witness the variability between animals in response to inflammation.

In Figure 31 we show the treatment groups results compared with the scramble siRNA treated group.
Figure 31. TNf-α level in macrophages extracted from the peritoneal cavity of treatment groups of mice compared with the scrambled group.

The level of TNF-α siRNA is shown, as normalized against β-actin. The Form 1, made with HA-C6:HA-PEI:HA-PEG 5:2:1 and loaded with cholesterol siRNA, results in an average silencing effect of 60% on TNF-α gene in the peritoneal cells. There is an animal out of four in the group that does not respond to the treatment. If we consider this animal as an outlier, the result is significant at α=0.05. We believe that 0.5 mg/kg LPS is a low dose, which is prone to increasing the variability to LPS response among animal subjects. The Form 2, made with HA-C6:HA-PEI:HA-PEG 3:2:1 results in an average silencing effect of 69%, which is significant at α=0.05 (p=0.039). The Form 3, made the same way as Form 2 except for, it encapsulates non-modified siRNA, results in 20% silencing, which is significant at α=0.1, (p-value= 0.088).

4.4 Conclusions

After recruiting macrophages in the peritoneal cavity, the TNF-α level in peritoneal macrophages was down-regulated, when the cholesterol modified siRNA was encapsulated in HA based polymer. This data indicates that at least two formulations out of three that we have selected from the in vitro screening are efficacious in reducing the mRNA level of TNF-α in peritoneal macrophages. We will select the Form 1 to use for the up-coming experiments, where
higher LPS values might be used. The data shown in this chapter is paired with the cytokines level in blood serum of the same animals. This data is shown in Chapter 6.
CHAPTER 5

BIODISTRIBUTION OF HYALURONIC ACID NANOPARTICLES

5.1 Introduction

Efforts in delivering drugs into cells and tissues have led to development of various vehicles particularly, nanotechnology has found immense improvement [50]. Many delivery vehicles might have undesired cell and tissue distribution. On the other hand the serum half-life might be unfavorable. Almost every drug development process requires special studies that evaluate the biodistribution and pharmacokinetics of the drug. Usually molecular imaging is very helpful and straightforward in tracking the drug final destination and provides helpful information in the steps needed for drug formulation specific improvement [51] [52]. For the purposes of this thesis work, we aimed quantifying the siRNA delivered into peritoneal macrophages. Since the cholesterol modified siRNA is reported to have a wide spread distribution, we checked the benefit of having a hyaluronic acid (HA) polymer nanoparticle to delivering the cholesterol modified siRNA into peritoneal macrophages. Besides the quantitative tracking of the siRNA distribution in cells and tissues, we aimed performing molecular imaging in order to visually show the whereabouts of the HA Cy 5.5 labeled nanoparticle encapsulated with siRNA.

5.2 Materials and methods

In order to quantify the nanoparticle distribution we traced the active strand of our siRNA by performing a qPCR assay [53]. In Figure 32 is shown the experimental set-up for this experiment.
Figure 32. The experimental design for the nanoparticle biodistribution experiment.

Figure 33. Stem-Loop qPCR method for siRNA quantification in cells, plasma and tissues. 1. Stem-loop RT primer binds to the 3’ end of the RNA. 2. RT product quantified: forward primer, reverse primer, TaqMan probe dye-labeled [53]

The siRNA nanoparticle was injected in the peritoneum then tissues and cells were extracted from the peritoneum. The active strand of the siRNA was quantified using a quantitative PCR method. This method that we use here to quantify the siRNA in tissues was at
first introduced in 2005 for quantifying the microRNA in tissues [53]. Similarly, the same protocol is adapted for the siRNA quantification, and has been extensively studied and optimized for pharmacokinetic and pharmacodynamic studies purposes at Alnylam Pharmaceuticals (Cambridge, MA). Briefly, we lysed the cells and tissues using triton 0.25% and heating up at 95°C. This process is supposed to disrupt cell and tissue membranes and release all proteins and the total messenger RNA from the cells. Then RT is performed to transcribe the combined DNA, followed by the PCR step. Specific primers and probes were designed for the TNF-α target. The method is known to be biased to the full length active RNA amplification; however some shorter mer-s might also amplify.

5.3 Quantitative assessment of siRNA biodistribution in peritoneal macrophage cells and tissues with use of qPCR technique tracing siRNA

In order to be able to quantify the siRNA of interest, we specifically designed forward primer, the stem-loop RT primer, and the probe which assure the specificity of the assay. In picture 33 we showed the principle of the method [53]. In Figure 34 is shown the designation process for the specific primers and probes needed to perform this protocol. The qPCR results were read on ABI 7900 Lite Cycler, based on manufacturer’s instructions.

**TNF-α**

**Antisense** AAGUUeAGuAGAcAGAAGAdTsdT

\[
gcccAAGTTCA\text{CTAGACAG, forward} \quad \text{AAGTTCA\text{CTAGACAGAAGATT}}\text{TTC\text{AGGTGTTCACTGATCGACTGACTTTCCCG}}
\]

<table>
<thead>
<tr>
<th>Antisense</th>
<th>TCGTAAAGGACATATTCCCGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem loop</td>
<td>GTCGTATCCAGTGCAAGGGTGTTCCGAGGTATTCGGATACAAGCcactctt</td>
</tr>
<tr>
<td>Forward</td>
<td>gcccAAGTTCA\text{CTAGACAG}</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGCA\text{GGGTTCCGAGGT}</td>
</tr>
<tr>
<td>Probe</td>
<td>5’-TGGATACGACcactctt</td>
</tr>
</tbody>
</table>


Figure 34. The process of primers and probes designed for the RT-PCR method of quantification of siRNA in cells and tissues.

5.4 Results and discussions

Below we show the results for the peritoneal cells, plasma, liver, kidney, spleen. The results are normalized against gram tissue, or gram protein respectively as required. A standard curve is built in duplicate for every tissue/cell where the blank matrix is used from the animals of the control untreated group. This way, the background reading of the Ct values taken from control animals’ tissues used in quantitative PCR, is subtracted from the one measured from the treated animals. This way the result which is shown, is calibrated based on the values from the control animals of the untreated group. In figure 35, is shown the siRNA level trend with relation to time, in peritoneal cells. We dosed the animals with 2 mg/kg siRNA encapsulated in HA polymer, so we injected an average 40 µg siRNA per animal.

![Standard Curve Peritoneal Cells](image)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>2</th>
<th>4</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA Full Length a-s (µg/mg protein)</td>
<td>0.3</td>
<td>1.0</td>
<td>0.6</td>
<td>1.1</td>
</tr>
<tr>
<td>St. Deviation</td>
<td>±0.2</td>
<td>±0.3</td>
<td>±0.3</td>
<td>±0.2</td>
</tr>
</tbody>
</table>
Figure 35. siRNA level in peritoneal cells. Top: Standard curve made using known concentrations of siRNA in PBS. Result normalized versus total cell protein (Total protein determined by the bicinchoninic acid assay, Thermo Scientific. Results not shown). Middle: The corresponding table of numerical values.

In Figures 36, 37, 38, 39 we show respectively the siRNA level measured in plasma, liver, kidney, and spleen.

<table>
<thead>
<tr>
<th>Time</th>
<th>2H</th>
<th>4H</th>
<th>24H</th>
<th>48H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Conc in ng/mL</td>
<td>1784.3</td>
<td>0.5</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Std Deviation</td>
<td>±1438.9</td>
<td>±0.3</td>
<td>±0.1</td>
<td>±0.0</td>
</tr>
</tbody>
</table>
Figure 36. siRNA level in plasma. **Top:** Standard curve made using known concentrations of siRNA in plasma from control-untreated animals. **Middle:** The corresponding table of numerical values.

<table>
<thead>
<tr>
<th>Time</th>
<th>2H</th>
<th>4H</th>
<th>24H</th>
<th>48H</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA Full Length Liver Conc (ng/g)</td>
<td>2.6</td>
<td>7.0</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>St Dev</td>
<td>±0.5</td>
<td>±1.2</td>
<td>±0.5</td>
<td>±0.5</td>
</tr>
</tbody>
</table>
Figure 37. siRNA level in liver tissue. **Top:** Standard curve made using known concentrations of siRNA in liver tissue lysate from control-untreated animals. **Middle:** The corresponding table of numerical values.

<table>
<thead>
<tr>
<th>Time</th>
<th>2H</th>
<th>4H</th>
<th>24H</th>
<th>48H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc/ Kidney (ng/g)</td>
<td>0.3</td>
<td>0.4</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>St Dev</td>
<td>±0.1</td>
<td>±0.3</td>
<td>±0.1</td>
<td>±0.0</td>
</tr>
</tbody>
</table>
Figure 38. siRNA in kidney tissue. **Top**: Standard curve made using known concentrations of siRNA in kidney tissue lysate from control-untreated animals. **Middle**: The corresponding table of numerical values.

<table>
<thead>
<tr>
<th>Time</th>
<th>2H</th>
<th>4H</th>
<th>24H</th>
<th>48H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc/ Spleen (ng/g)</td>
<td>21.3</td>
<td>2.9</td>
<td>24.2</td>
<td>3.4</td>
</tr>
<tr>
<td>St Dev</td>
<td>±19.7</td>
<td>±2.8</td>
<td>±16.8</td>
<td>±0.4</td>
</tr>
</tbody>
</table>

\[ y = -3.1919x + 24.858 \]
\[ R^2 = 0.9905 \]
Although it is not easy to make an accurate mass balance of the injected drug, from the data we can see that the highest drug concentration is found in the peritoneal cells, as compared to other tissues and plasma. In table 12, we show average siRNA level in peritoneal cells to compare nanoparticles injected siRNA versus naked injected siRNA.

**Table 12.** Average siRNA values of naked siRNA versus formulated siRNA in peritoneal cells

<table>
<thead>
<tr>
<th>Time point (Hours)</th>
<th>Naked siRNA (µg/mg total cell protein)</th>
<th>Nanoparticle siRNA (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$0.2 \times 10^{-1}$</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>Unmeasurable</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>$0.1 \times 10^{-1}$</td>
<td>0.6</td>
</tr>
<tr>
<td>48</td>
<td>$0.9 \times 10^{-2}$</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The data indication complies with reported findings from the literature based on previous independent studies: the cholesterol conjugated on the siRNA, represents a delivery vehicle and delivers the siRNA in cells and tissues. However, this type of delivery is characterized by a wide nonspecific distribution. Interestingly, we observe that the amount of the RNA measured in the
cells is inconsiderable for the naked siRNA delivery compared to the one delivered by means of encapsulated siRNA in nanoparticle. This means that, using the combinatorial approach, we successfully deliver the siRNA preferably into the peritoneal cells. More importantly, we measure considerable amounts of the siRNA in the cells until 48 hours after injection, if nanoparticle encapsulated system is used. However, this data does not show much about the mechanism of nanoparticle delivery, and data corroboration with silencing efficacy is needed.

5.5 Conclusions

With means of a quantifying qPCR method, we have determined the amount of siRNA which is found in peritoneal cells, plasma, and liver, kidney, and spleen tissues. The results show that peritoneal cells take the nanoparticle up as early as 2 hours after injection in the peritoneal cavity. More importantly, the data indicates that the nanoparticle system is helpful in delivering the siRNA preferably into peritoneal cells, as compared to other organs in the peritoneum, when the combinatorial approach is used the cells take up significantly higher siRNA as they do when the naked siRNA is delivered. These findings further complete the information taken from the imaging studies.

5.6 In vivo whole animal imaging assessment of nanoparticle distribution

As described in 5.4, we are confident that the nanosystem we are applying delivers the siRNA into the peritoneal cells. We find siRNA in the liver, spleen, and kidney as well. We aimed to visualize the nanoparticle journey into the peritoneum therefore we had two options to achieve this goal. Either siRNA or polymer could be labeled with fluorescent dye, and pictures can be taken with use of in vivo imaging system. We chose to label the HA polymer with Cy5.5 fluorescent dye. Cyanine 5.5 is a reactive dye which is conjugated to proteins, oligonucleotides and polymers via NHS ester bond. The excitation maximum wavelength is 673nm, and
maximum emission wavelength is 707 nm. After encapsulation of 2mg/kg siRNA into the nanoparticle made with Cy5.5 labeled HA –PEI polymer, we anesthetized the animals at the designated time point and took images of the whole animal body, harvested the peritoneal lavage, then extracted liver, kidney and spleen tissue. The images were taken at 3H, 6H, 18H, and 24H time points on an IVIS instrument based on manufacturer’s instructions. In addition to whole body pictures, the peritoneal cells were extracted and fixed/ stained in chamber slides for microscopy images at 3H, 6H, 18H, and 24H. We also extracted liver, kidney and spleen from the animal at 18H time point, in order to visualize the nanoparticle uptake into these organs. The peritoneal cells were stained and fixed into chamber slides to be able to be seen under microscopy. After peritoneal lavage, cells were spun out and reconstituted twice in DPBS in order to remove peritoneal fluids. After two rinses, the cells were reconstituted in DMEM complete medium containing 10% FBS, and incubated at 37°C for 30min to 45 min in order to adhere in two well chamber slides. The adhering cells would mainly be macrophages, which then were rinsed with DPBS twice. Cells surface was blocked with Bovine Serum Albumin (BSA) solution for 10 min at room temperature then rinsed twice with DPBS. The adhered cells were then stained with CD-11-FITC antibody for 1 hour at room temperature, in order to detect macrophages specifically on microscopy. At the last step the cells were fixed with use of paraformaldehyde for 10 min, and after rinsing with PBS were cultured in mounting medium conjugated with DAPI dye. The chamber was detached from the slide and the slide was covered with microscope cover slips until microscopy.

5.6.1 Results and discussions

In Figure 40 below we are showing the images of intraperitoneal injected mice with labeled nanoparticle after 3 hours, 6 hours, 18 hours, and 24 hours.
Figure 40. Whole body pictures taken from mice at 3H, 6H, 18H, and 24 hours after HA-PEI Cy5.5 labeled injection.

At 18 hours we extracted liver, kidney, and spleen from the treated animals with HA-PEI_Cy5.5 nanoparticle, and untreated animals. The images are shown in Figure 41.

Figure 41. Liver, kidney, and spleen fluorescence from treated animals with HA-PEI-Cys5.5 nanoparticle treated animals, compared to naïve animals.

The images show the distribution of the labeled nanoparticle into the peritoneum with respect to time after injection. In Fig 40, we observe that the particle is distributed fairly rapidly in the peritoneum during the first 6 hours. At 18 hours and 24 hours, the fluorescence fades out. It is well known fact that the HA is synthetized and metabolized following a regular cycle in the organism, so it is not surprising if the labeled HA we injected in animals metabolizes in the liver, and gets cleared through kidneys, which likely explains why liver and kidney fluoresce at 18 hours after injection. It is also likely that the peritoneal macrophages start migrating, taking the nanoparticle they uptake away from the peritoneum. However, the local injection in the
peritoneum might have led to some uptake in the liver and kidney. Not much fluorescence, if at all, is measured in the spleen at 18 hours. In Figure 42, we show cell trafficking images taken on confocal microscopy at 3H, 6H, 18H, and 24H after nanoparticle injection. The images indicate that the nanoparticle uptake starts as early as 3 hours, but it reaches a peak at 18 hours, and plateaus out at 24 hours after injection. If compared with whole body images the data indicates that the fluorescence starts fading out at 18 hours, right when the uptake reaches a peak in peritoneal macrophages. This data respect our findings from the silencing studies where we only see silencing effect at after 24 hours after injection with siRNA.

![Figure 42](image)

**Figure 42.** Images taken from stained mouse peritoneal macrophages after injection with HA-Cy5.5 at 3 hours, 6 hours, 18 hours, and 24 hours, respectively from left to right.

### 5.5.2 Conclusions

Using both imaging and siRNA quantification, we have shown that the nanosystem encapsulating siRNA is taken up in peritoneal cells early after injection in mice. The peak seems to be reached at 18 hours therefore it is after this time point that the RNAi is more likely to start its effect. Although the whole body images simply visualize what we would expect, they still comply with the cell images. At 18 hours the fluorescence intensity starts decreasing, while the cell uptake increases. Clearly, macrophages seem to be taking the nanoparticle fairly consistently.
CHAPTER 6

IN VIVO STUDIES OF SYSTEMIC INFLAMMATORY RESPONSE DUE TO ANTI-TNF-α siRNA TREATMENT

6.1 Introduction

An infection or tissue injury is associated with an inflammatory response. Pattern recognition receptors (PRRs), directly or indirectly detect pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acids, lipoproteins, and carbohydrates. Cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL1), promote extravasation of leukocytes leading a process of chain events, which creates a storm of cytokines (fig 43) [54]. We aimed to observe the impact that the silencing of the TNF-α gene in macrophages, had in breaking of the cytokine storm. For this purpose, we measured the level of TNF-α in macrophages at the site of insult then, we measured the level of a pool of pro and anti-inflammatory cytokines in the blood serum.

Figure 43. Cells and mediators of the inflammatory response. Molecules derived from plasma proteins and cells in response to tissue damage or pathogens mediate inflammation by
stimulating vascular changes, plus leukocyte migration and activation. Granulocytes include neutrophils, basophils, and eosinophils.

6.2 Systemic anti-inflammatory response relative to local TNF-alpha silencing in peritoneal macrophage

6.2.1 Materials and methods

In section 6.1.1.1 of this chapter, we will extend the results of the work which we have described in Chapter 4, section 4.2. After following an experimental set-up described in that chapter, we blooded the animals on the day of necropsy and span the blood samples down after leaving them at room temperature for 2 hours. The serum samples were then stored at -20°C until further analyzing. In order to observe the trend of a panel of cytokines after TNF-α silencing in the macrophages of the peritoneum, we analyzed the cytokine concentration of the animals’ serum samples. The analytical method we used was the same as the one we described in Chapter 3, section 3.4 in an in vitro experiment using J774 A1 cell line. As described earlier for the in vitro assay, 25 cytokines using this high-throughput approach. Practically, it is difficult to achieve accurate numbers for all the analytes using a unique dilution, especially when the expected ranges are not well known for the 25 cytokines of the panel. The assay has a measurement range of: from 0 pg/mL to 10,000 pg/mL. It is worthwhile to mention that for some cytokines the values were high beyond the range of detection. If a dilution of 10:1 did not help to measure an accurate value, we assumed that their levels were increased beyond the value of 10,000 pg/mL.

In the section 6.1.1.2 below, we will discuss the inflammatory response relative to TNF-α silencing effect in peritoneal macrophages, the later regulated by exposing animals to escalated doses of LPS of 1, 2, and 3 mg/kg, while injecting the same siRNA dose of 2 mg/kg. In figure 44 is shown the in vivo experimental set-up. Similarly with what is described in Chapter 4, we used
the same method to purify peritoneal cells collected from animals on the day of necropsy. Also, we followed the same qPCR protocol to quantify the mRNA level in peritoneal macrophages, as well as we used the same protocol to quantify the cytokines in blood serum of animals.

![Figure 44](image)

**Figure 44.** Experimental design of the in vivo experiment using escalating dose of LPS at 1 mg/kg, 2 mg/kg, 3 mg/kg.

6.2.2 **Results and discussions**

6.2.2.1 **Results and discussions for the experiment described previously**

In Figure 45, we show the cytokine expression profile in blood serum of animals as a result of intraperitoneal LPS induction, with the one in blood serum of untreated group of animals.
Figure 45. Cytokine expression in blood serum for animals treated with TG 3 mg/kg then 0.5 mg/kg LPS. Blue bars represent treated animals with TG, then LPS; red bars represent untreated animals.

The data indicates that the induction using 0.5 mg/kg LPS is clearly affecting the panel of cytokines down the stream from TNF-α stimulation. As TNF-α is upregulated, so do the majority of cytokines in the blood stream, as shown from the serum samples analyzed at 24 hours after LPS intraperitoneal injection. These would be the level of cytokines in the blood serum if the animals induced with 0.5 mg/kg LPS, were not treated with siRNA nanoparticle.

In Figure 46 are shown the results for the panel of cytokines in the blood serum of treated animals with anti-TNF-α siRNA (three formulations), versus the cytokines in the blood serum of treated animals with scramble siRNA.
In general, we observe down-regulation of the cytokines in blood serum in the treated groups with TNF-α siRNA nanoparticle, especially for FORM 1 and FORM 2 nanoparticles. Let’s recall here that we measured down-regulated values for the TNF-α in the macrophages collected from the peritoneum in those animals treated with nanoparticle, versus the animals treated with scramble siRNA, as shown in Chapter 4. Elevated levels for IL-6 and KC were observed in the animal group treated with FORM 2. This is a surprise to us because the results from the Form 1 and Form 3 do not synchronize with Form 2. However, for the purposes of this experiment, we can generalize the down-regulating result for the majority of cytokine level in blood serum, and plan to observe this effect carefully in the future experiments. We cannot conclude these results represent a cause-effect relationship between TNF-α down-regulation in the peritoneal cavity and cytokine level in the blood serum. This result is obtained for one time point only and one single dose was used.
6.2.2.2 Results from in vivo experiments using escalating LPS dose

In support to our main hypothesis, we performed IN VIVO experiments to check for the silencing efficacy, and monitor the effect in the downstream cytokines. We aim to further investigate the relationship between TNF-α level in peritoneal macrophages, and cytokines in the blood serum, 48 hours after LPS injection. We aimed to look deeper into physiology of an expanding inflammation, and its alterations when TNF-α is altered with use of siRNA therapy. We used increasing LPS doses from 1 mg/kg to 3 mg/kg in the peritoneum and treated the animals with 2 mg/kg siRNA nanoparticle encapsulated drug simultaneously. With full intent, we did not change the siRNA dose, in order to be able to monitor the latitude of cytokines in the blood serum in varying LPS concentrations. In figures and tables below are shown the results for TNF-α in peritoneal macrophages and cytokines in the blood serum.

![Graph showing mRNA TNF-α/b-actin levels](image)

**Figure 47.** TNF-α in peritoneal macrophages, and cytokine levels measured in blood serum of animals treated with 1 mg/kg LPS; 2 mg/kg siRNA at 48 hours after injection.
Table 13. Cytokine level in blood serum in animals treated with 1 mg/kg LPS and 2 mg/kg siRNA

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Value in naïve mice (pg/mL)</th>
<th>Treated mice (pg/mL)</th>
<th>Treated mice/ scramble 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>200±0.7</td>
<td>9432.8 ±335.6</td>
<td>8814.9±261.6</td>
</tr>
<tr>
<td>IL-1α</td>
<td>162±13.2</td>
<td>321.2±17.7</td>
<td>256.4±9.5</td>
</tr>
<tr>
<td>IL-5</td>
<td>3.2±4.2</td>
<td>12.8±0.6</td>
<td>4.8±0.4</td>
</tr>
<tr>
<td>IL-6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-10</td>
<td>0</td>
<td>2.2±0.0</td>
<td>8.9±0.2</td>
</tr>
<tr>
<td>IL-13</td>
<td>54.9±7.8</td>
<td>64.7±3.7</td>
<td>263.9±10.8</td>
</tr>
<tr>
<td>IL-15</td>
<td>0</td>
<td>11.42</td>
<td>34.8</td>
</tr>
<tr>
<td>IP-10</td>
<td>62.3±14.4</td>
<td>267.6±5.2</td>
<td>205.8±25.4</td>
</tr>
<tr>
<td>KC</td>
<td>45.7±41.9</td>
<td>118.7±5.8</td>
<td>52.4±0.5</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0</td>
<td>85.6±1.9</td>
<td>46.0±2.9</td>
</tr>
<tr>
<td>MIP1-a</td>
<td>0</td>
<td>37.2±1.5</td>
<td>17.1±4.5</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RANTES</td>
<td>13.6±6.1</td>
<td>32.7±1.00</td>
<td>21.6±0.9</td>
</tr>
<tr>
<td>TNF</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TNF-α level in macrophages from animals treated with 2mg/ LPS, 2 mg/kg siRNA is shown in Fig. 48.

![mRNA TNF-α/b-actin](image)

**Figure 48.** TNF-α in peritoneal macrophages , and cytokine levels measured in blood serum of animals treated with 2 mg/kg LPS and 2 mg/kg siRNA.
Table 14. Cytokine level in blood serum in animals treated with 2 mg/kg LPS and 2 mg/kg siRNA.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Value in naïve mice (pg/mL)</th>
<th>Treated mice (pg/mL)</th>
<th>Treated with scramble (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>200±0.7</td>
<td>13612.8±14.3</td>
<td>16575.5±158.7</td>
</tr>
<tr>
<td>IL-1a</td>
<td>162±13.2</td>
<td>150.8±11.8</td>
<td>151.8±10.9</td>
</tr>
<tr>
<td>IL-5</td>
<td>3.2±4.2</td>
<td>7.2±0.1</td>
<td>9.6±0.1</td>
</tr>
<tr>
<td>IL-6</td>
<td>0</td>
<td>10.9±0.7</td>
<td>24.8±1.4</td>
</tr>
<tr>
<td>IL-10</td>
<td>0</td>
<td>4.4±0.5</td>
<td>3.37±1.4</td>
</tr>
<tr>
<td>IL-13</td>
<td>54.9±7.8</td>
<td>98±2.7</td>
<td>34±10.2</td>
</tr>
<tr>
<td>IL-15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IP-10</td>
<td>62.3±14.4</td>
<td>206.5±61</td>
<td>184.2±17.4</td>
</tr>
<tr>
<td>KC</td>
<td>45.7±41.9</td>
<td>41.2±2.7</td>
<td>108.1±4.5</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0</td>
<td>24.1±2</td>
<td>39.7±1.2</td>
</tr>
<tr>
<td>MIP1-a</td>
<td>0</td>
<td>16.3±0.8</td>
<td>11.48±4.0</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>0</td>
<td>13.4±1.9</td>
<td>46.4±1.0</td>
</tr>
<tr>
<td>RANTES</td>
<td>13.6±6.1</td>
<td>19.5±0.8</td>
<td>16.3±0.7</td>
</tr>
<tr>
<td>TNF</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results from animals treated with 3 mg/kg LPS are shown below in figure 49.

![mRNA TNF-α/b-actin](image)

**Figure 49.** TNF-α in peritoneal macrophages, and cytokine levels measured in blood serum of animals treated with 3 mg/kg LPS and 2 mg/kg siRNA.
Table 15. Cytokine level in blood serum in animals treated with 3 mg/kg LPS and 2 mg/kg siRNA.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Value in naïve mice (pg/mL)</th>
<th>Treated mice (pg/mL)</th>
<th>Treated with scramble (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>200±0.7</td>
<td>3347.4±689.5</td>
<td>36657.4±270</td>
</tr>
<tr>
<td>IL-1α</td>
<td>162±13.22</td>
<td>205.73±13.12</td>
<td>151.81±15.8</td>
</tr>
<tr>
<td>IL-5</td>
<td>3.2±4.2</td>
<td>4.1±0.2</td>
<td>9.6±0.4</td>
</tr>
<tr>
<td>IL-6</td>
<td>0</td>
<td>10.7±1.0</td>
<td>17.2±0.4</td>
</tr>
<tr>
<td>IL-10</td>
<td>0</td>
<td>13.7±1.8</td>
<td>26.6±1.0</td>
</tr>
<tr>
<td>IL-13</td>
<td>54.9±7.8</td>
<td>34.3±2.7</td>
<td>42±14.9</td>
</tr>
<tr>
<td>IL-15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IP-10</td>
<td>62.3±14.43</td>
<td>205.54±24.26</td>
<td>155.44±12.47</td>
</tr>
<tr>
<td>KC</td>
<td>45.7±41.9</td>
<td>32.3±2.9</td>
<td>85.8±8.1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0</td>
<td>36.2±3.5</td>
<td>69.3±1.1</td>
</tr>
<tr>
<td>MIP1-a</td>
<td>0</td>
<td>19.1±1.1</td>
<td>22.9±8.9</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>0</td>
<td>46.6±3.7</td>
<td>61.1±1.1</td>
</tr>
<tr>
<td>RANTES</td>
<td>13.6±6.1</td>
<td>10.4±1.4</td>
<td>20.9±0.3</td>
</tr>
<tr>
<td>TNF</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The data indicates that the TNF-alpha local silencing in the peritoneum has an impact on values of several cytokines down the stream in the blood serum, which also down-regulate their levels. These cytokine levels are shown in red on the respective tables. Surprisingly, the picture becomes clearer as the silencing of the TNF-alpha becomes moderate and drops from 82% in the case of 1 mg/kg LPS induction, to 17% in the case of 3 mg/kg LPS induction. We explain this with the fact that TNF-alpha is an important mediator in inflammation and sepsis, and the cytokines in its pathway are down regulated down the stream if TNF-α itself is also down-regulated. However, if we knock down the TNF-alpha like in the case of the 1 mg/kg LPS induction, then other mediators such as IL-1 a and IL-1β are still playing their role in the presence of LPS, keeping the signaling going down their pathway. Therefore, we do not see the down-regulation of the cytokine storm if TNF-α is deeply knocked down. Apparently, if TNF-α is moderately knocked down, the same cytokines are significantly down regulated (α=0.05).
Based on these results, we would like to stress out that the TNF-α level should be titrated in a way that allows it to still play its pro-inflammatory role, while breaking its action to a certain extent, i.e., here we propose 20% knockdown. Knowing the important role of TNF-alpha in inflammation, from this data we get the important message that a moderate silencing effect is more efficient in cytokine cascade silencing then a complete knock down would be. So, we should dose our siRNA at reasonable levels that affect 20-30% of the TNF-alpha in macrophages in peritoneum, which allows for the remained TNF-alpha to continue and play the pro-inflammatory role during sepsis.

6.2.3 Conclusions

Using the local inflammation model we have concluded that the TNF-α level in peritoneal macrophages down-regulate if the animal is treated with anti-TNF-α siRNA. More importantly this down-regulation in TNF-α level, seems to be followed by the down-regulation of a panel of cytokines which otherwise if left untreated increase their levels compared with their normal ones in untreated animals. However, an important finding is that the TNF-α level should not down-regulate further than 20% of its normal values, which prompts for a titration of the siRNA therapy effect in order to reach a positive effect. This is in accordance with the fact that TNF-α is an important mediator of inflammation and its total absence harms the host immune response, whereas a moderate down-regulation helps the silencing of the cytokine storm, while at the same time allowing TNF-α to play its important role under controlled levels.
6.3. In vivo measuring the signaling events at the site of infection

6.3.1 Introduction

Cells of innate immune system, such as macrophages, fibroblasts, mast cells, and dendritic cells respond to the infection or tissue injury through intensive cytokine release [55]. This cytokine release shapes the slower, lymphocyte-mediated adaptive immune response. In the previous Chapters we have shown data on how TNF-α silencing on peritoneal macrophages, likely affects the level of a panel of pro and anti-inflammatory cytokines in the systemic system [54]. In support to **Aim 4**, and in order to observe the cytokine behavior at the local site, we designed an *in vivo* experiment in order to collect concentrated peritoneal lavage at different time points. This data might be important to better understand the crosstalk among cells in the peritoneum, when LPS is injected at 3 mg/kg. An approach like this is also helpful to measure the trend of cytokine release at the local site relative to time, and understand how certain cytokine level correlates with the TNF-α level released at the site.

6.3.2 Materials and methods

The experimental set up was such as the thioglycollate is injected in the peritoneal cavity to promote macrophage recruitment at the site, on the first day of experiment. By day 3, LPS at 3mg/kg is injected along with either active siRNA formulation, or scramble siRNA formulation at 2 mg/kg. At the designated time point of 2H, 6H, 15H, 24H, and 48H, the peritoneum was rinsed with only 1 mL of PBS, in order to be able to pipet out a concentrated enough solution that would generate readable values for the cytokines. Similarly, we have used a Milliplex kit of 4 cytokines, which are known to play an important role in the first response to inflammation.

6.3.3 Results and discussions
In Fig 50, we have shown the cytokine levels in the 1x PBS solution form peritoneum, for the TNF-α, IL-6, IL-1b, IL-1a.

**Figure. 50.** Cytokine level of released in the peritoneum by macrophages, at different time points. From top to bottom: TNF-α; IL-6; IL-1b

Based on this data, there is a maximum level of TNF-α excreted at around 6 H, then the level drops to as low values as 3,4 pg/mL. Although there is a trend showing lower levels for the
TNF-α excreted by cells in the peritoneum of animals treated with active siRNA, the result is not significant. This is due to the fact that not every subject animal reacts the same way to the LPS treatment, but also an earlier data point than 4 H should be monitored. The TNF-α is a fast responder. Looking at the IL-6 and IL-1b, we find that the cytokine release is significantly lower in the peritoneum of the animals treated with active anti-TNF-α siRNA, as compared to the scrambled ones. The IL-1a (not shown), does not represent a clear trend. Instead, for the IL-1a large variability is observed within the animals of the same group. It is a well-known fact that that IL-1a is an inflammatory mediator by itself, and it is not expected to be on the TNF-α pathway.

6.3.4 Conclusions

It is likely that the orchestrating role of TNF-α is played very early in the inflammation process, at the local site of inflammation. More importantly, even though we are able to measure elevated levels in LPS treated animals only until 6 hours after injection, we are able to find elevated cytokine level in the blood serum until 48 hours after LPS injections, in untreated animals with siRNA. The immune response is signaled from the molecular level, to the local site of injection, then into systemic circulation. Once the elevated cytokine flux reaches the systemic system, it needs time to turn back into the baseline level, even though the power of the local infection starts weakening. Measuring a pool of different cytokines becomes challenging if we consider that the time to peak for them is not the same.
CHAPTER 7

ASSESSMENTS OF THERAPEUTIC EFFICACY OF THE RNAi IN LPS-MODEL OF SEPSIS THROUGH ANIMAL SURVIVAL

7.1 Introduction

Early diagnosis of sepsis has continuously been a challenge, given that early symptoms can be misleading. There is potential risk that by the time doctor realizes the patient has sepsis syndrome, it is already too late. Although earlier diagnosis might be of enormous importance to cure sepsis, even in the earliest detections cases the patient’s survival becomes the main concern. Here we aim to develop experiments that monitor the survival benefit offered by the siRNA therapy, which moderately targets the TNF-α gene under septic conditions.

7.2 Materials and methods

7.2.1 Protocol of in vivo survival experiment

We aim to assess the survival benefit of our treatment. The first concern we needed to overcome was deciding about the LPS dose that we needed to use for sepsis trigger, in order to avoid anaphylaxis shock. Based on the literature data, it is reported that doses as high as 16-20 mg/kg were used in the past leading to a mortality of 100% within 48 hours [56]. For purposes of our thesis, we planned to use 10 mg/kg LPS dose in C57bl/6 male mice, 8 animals per group. Based on our previous studies where we used 2 mg/kg siRNA and 3 mg/kg LPS with an impact on the cytokine storm down-stream, we reasoned that for this LPS dose we need to use 7 mg/kg siRNA dose, administered by IP route. Mice were watched closely for their behavior and weight loss. When and where necessary they were euthanized appropriately and sacrificed by cervical
dislocation under isoflurane. From our experience with up to 3mg/kg LPS dose, it is indicated that mice do not exceeded 15% loss of the entire body weight. At 48 hours the mice start recovering, regain their active movements, and start feeding better. However, 10 mg/kg LPS dosing might trigger weight loss at or higher than 15%. Therefore, we observed the animals for the first 6 hours. At 3 hours after LPS administration, we injected Buprenorphine SC at 0.1 mg/kg in order to alleviate pain; then we administered Buprenorphine SC twice daily at 0.1 mg/kg for 2 days. The body weight was observed once a day and we sacrificed the animals under proper anesthesia, when they were showing extensive morbidity due to LPS. Animals were monitored three times daily after LPS treatment. The final end points used were 15% weight loss with morbidity. To statistically elaborate the data we used the SPSS software, Kaplan-Meier hypothesis test for survival, hypothesizing that the treatments are all the same. We aimed a level of significance $\alpha=0.05$.

### 7.2.2 Results and discussions

In Figures below, we show the results from the survival experiment elaborated on SPSS, expressed as survival probability using the Kaplan-Meier method for survival experiments. In Figure 51, we are showing the SPSS output for the Kaplan-Meier survival curves for 10 mg/kg LPS, and siRNA pretreatment with 7 mg/kg dosing.
Overall, if we pretreat animals with TNF-α siRNA, there is a significant survival benefit among septic animals challenged with 10 mg/kg LPS, versus septic animals with scramble control siRNA. This is attested by the p-values found by the software for the three areas of the plot: early time points, developing time, and later time points. All these p-values, are lower than 0.05 level of significance. This indicates that there is a difference between treatments, therefore a benefit to pretreating the animals with siRNA before challenging with LPS. However, if we induce sepsis at the same time with the siRNA injection, no survival benefit is observed. The SPSS output is shown in Figure 52 below.
Figure 52. SPSS output for the survival experiment using 10 mg/kg LPS dose and 7 mg/kg siRNA, n=7 as a simultaneous treatment approach (control group n=8). Chi-Square statistic is not large enough for the p value to be lower than 0.05, therefore the result is not significant. These two treatments are the same.

7.2.3 Conclusions

A survival benefit is evidenced in this experiment outcome, in the group of mice pretreated with siRNA. The approach of treatment with siRNA simultaneously with the LPS induction did not result in survival benefit, likely due to the fact that there is a shifting of RNAi and LPS effects. There were a couple of drawbacks in the way the experiment was performed. The dosing of LPS at 10 mg/kg seemed harsh. Also, using of buprenex injection twice a day might have accentuated hypothermia symptoms which are caused by sepsis anyways. We further confirmed the results in another experimental setup that corrected these drawbacks.
7.3 Survival benefit assessment using modified approach

7.3.1 Methods and materials

Based on our previous experiment where we injected 7 mg/kg siRNA and 10 mg/kg LPS, we reasoned that a LPS dose of 10 mg/kg administered by IP route is obviously high and mice undergo a fast onset of shock condition. This shock condition is not likely reversible, so considerable number of animals could not survive. We repeated the experiment, in order to assess the survival rate under milder septic conditions. Therefore, we injected 5 mg/kg dose for LPS and 3 mg/kg dose for the siRNA. We used a similar approach as for the previous experiment, and designed three groups of mice: 1) 10 mice pre-treated with siRNA, then challenged with LPS after 24 H; 2) 7 mice treated with LPS and siRNA at the same time (accidentally lost 3 mice over them hurting each other while fighting); and 3) 10 mice treated with scramble siRNA and LPS to use as a control group. Mice were watched closely for their behavior and weight loss every 6 hours during the first day, and then twice daily. The experiment lasted 6 days, after which time the survived animals were obviously recovering/ recovered. When and where necessary they were euthanized appropriately and sacrificed by cervical dislocation under isoflurane anesthesia. Therefore, we observed the animals frequently for the first 6 hours. We provided Tylenol 1.5 mg/mL in drinking water, and injected 1 mL of Saline lukewarm solution via IP, for two days after LPS injection, to account for interacting body weight loss with results. The body weight was observed once a day and we sacrificed the animals under proper anesthesia, when they were showing extensive morbidity and/or weight loss beyond 15% due to LPS, based on the end points determined on the protocol. The final end point was again 15% weight loss with morbidity.
7.3.2. Results and discussions

In Figure 53 is shown the SPSS elaborated result from the survival experiment using LPS 5 mg/kg as sepsis trigger. In this approach TNF-α siRNA was used at 3 mg/kg to pretreat the animals 24 hours before the LPS at 5 mg/kg was injected. In Figure 7.4 is shown the SPSS elaborated result from the survival experiment performed at the same time as the one shown in Fig 53, but in this case both siRNA and LPS were injected at the same time.

![Survival Functions](image)

- **Overall Comparisons**
  - Chi-Square df   Sig.
  - Log Rank (Mantel-Cox)  4.819  1   .028
  - Breslow (Generalized Wilcoxon)  4.420  1   .036
  - Tarone-Ware  4.637  1   .031

**Figure 53.** Survival result that shows SPSS output, when septic animals pretreated with siRNA were compared with the control group of mice, n=10. Significant level α=0.05.
Figure 54. Survival result that shows SPSS output, when septic animals treated with siRNA and LPS at the same time n=7, were compared with the control group of mice n=10. Significant level $\alpha=0.05$.

The result clearly shows that the siRNA treatment to target TNF-$\alpha$ in septic animals is beneficial with regard to survival rate, at a level of significance of 5%. However, the data indicates that septic conditions need to be set up, such as we do not create instant irreversible shock, represented here with 5mg/kg LPS dose. If more severe inflammation is set up, like the one represented here with the 10 mg/kg LPS dose, then the TNF-$\alpha$ siRNA treatment is beneficial in case the animals undergo pretreatment. Although, the simultaneous treatment with siRNA and LPS did not represent significant benefit, the entire experimental set up with 10 mg/kg confirmed one more time that TNF-$\alpha$ is an important player during inflammation and sepsis.

All results are summarized in Figure 55.
Figure 55. SPSS output showing results of the survival benefit to siRNA treatment, when 5 mg/kg LPS dose is used to trigger sepsis. The p-values in red are lower than 0.05, which indicates that the results are different from one another, so there is a benefit to siRNA treatment.

7.3.3. Conclusions

The TNF-α siRNA treatment results to be significant if 5 mg/kg LPS dose is used to trigger sepsis. TNF-α modulation at that step, where it is an important inflammation mediator, results in full recovery of almost 80% of septic animals, versus 30% full recovery of septic animals from the control group. However, the result achieved when a twice higher LPS dose was injected in animals, confirms that TNF-α is an important mediator and target during sepsis and inflammation. Another take from this experiment is that, siRNA therapy is feasible under septic condition provided that the siRNA formulation is optimal for the RNAi to happen as soon as
possible. The synchronization between RNAi and TNF-α peak is key to the success of this therapy.

As might be expected one may ask: “What is the mechanism of siRNA treatment that leads to therapeutic efficacy as shown here?”

Based on the compiled data we are confident that the siRNA, which is taken up by immune cells at the site of triggered infection, modulates the TNF-α gene. The later in turn, keeps playing its role in inflammation since we only modulate close to 20% of its expression, on the other hand the TNF-α signaling pathway is slowed down by this modulation, which retards the cytokines flux in becoming a powerful uncontrollable storm. This is likely to help maintain the balance between SIRS and CARS during the septic condition that we have created. More importantly, the systemic flux of cytokines is broken, which creates conditions for the animals to survive, and ultimately recover.
CHAPTER 8
PRELIMINARY SAFETY ANALYSES OF HA POLYMER SYSTEM UPON INTRAPERITONEAL ADMINISTRATION

8.1 Introduction

To support our specific aim 5, we aimed monitoring of the safety of our drug. Many drug side effects are mediated by the immune response and TNF-alpha and IFN-γ are important markers to any drug interaction with the immune system [57] [58]. Previous studies have found no adverse effects triggered by the HA-PEI polymer [38]. On the other hand it is known that the siRNA would trigger immune response through TLR 7 and elevated IFN-γ has shown to be an important biomarker to confirm it [59]. It is also reported that monitoring antibody production following a drug treatment, it is a helpful tool to discover drug adverse effects [60]. For the purposes of this thesis work and to achieve the goal, we focused on detection of cytokines such as TNF-alpha and IFN-γ which are well-known as first responders to immune stimulation, even before the antibodies are produced.

8.2 Materials and Methods

The figure 56 sketches the simple setup of the in vivo experiments, were animals were exposed to extra high doses of polymer, siRNA, and nanoparticle. These doses are not the typical amounts that we inject the animals with, however we aimed to check on the limit that would trigger an immune response to the drug itself, while monitoring the polymer at very high atypical doses. The animals were blooded at 15 hours, a shorter time frame than 24 hours. We performed cytokine measurement assay to measure the cytokine level in the blood serum of animals, with use of Milliplex kit.
8.3 Results and Discussions

Table 16 displays the readings of the instruments for the IFN-γ, and TNF-α in animals’ blood serum.

Table 16. Software output for TNF-α and IFN-γ

<table>
<thead>
<tr>
<th>Group</th>
<th>IFN-γ</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reading</td>
<td>Interpretation</td>
</tr>
<tr>
<td>2 mg/kg siRNA (NP)</td>
<td>OOR</td>
<td>Out of range below</td>
</tr>
<tr>
<td>5 mg/kg siRNA (NP)</td>
<td>OOR</td>
<td>Out of range below</td>
</tr>
<tr>
<td>10 mg/kg siRNA (NP)</td>
<td>OOR</td>
<td>Out of range below</td>
</tr>
<tr>
<td>30 mg/kg siRNA (NP)</td>
<td>OOR</td>
<td>Out of range below</td>
</tr>
<tr>
<td>85.5 mg/kg Polymer</td>
<td>OOR</td>
<td>Out of range below</td>
</tr>
<tr>
<td>30 mg/kg naked siRNA</td>
<td>OOR</td>
<td>Out of range below</td>
</tr>
<tr>
<td>Naïve Mice</td>
<td>OOR</td>
<td>Out of range below</td>
</tr>
</tbody>
</table>

The result shows that there is no indication of immune response to drug administration. More importantly even the highest doses administered do not seem to trigger any adverse effects.
This finding is in accordance with the results from the past studies of Dr. Amiji’s group where no
toxicity has been observed that relates to the HA polymer nanoparticles administration. In
Appendix 1, the standard curves and the results generated by the control reagents are shown to
testify for the reliability of the results.

**Concluding remarks**

One of the most challenging aspects of sepsis treatment has been lack of a specific
therapy. Sepsis is one of those syndromes that advance quickly over the course of the
development. While the use of antibiotics is a golden standard for curing those cases when a
pathogen is present, not much is available to cure the sterile sepsis. The success of curing sepsis
in clinic is low, as much as 40% of septic patients would die. A “one fits all” therapy is not
feasible for sepsis. For example, in the case of trauma or injury, no antibiotic would help to cure
the sepsis itself, unless it is needed when an infection overlaps it. In the current study, a
combinatorial approach of nano size range was developed to target macrophages and modulate
their intracellular TNFα level. This combinatorial approach demonstrated significant survival
benefit in septic animals where the TNF-α was the main inflammation mediator. In order to
confirm the efficacy of the system, a local peritoneal inflammation model using LPS was
evaluated in mice. We showed that the siRNA therapy might become a powerful tool in curing
sepsis, if targets are chosen carefully, and its pathway is traced down the stream of the gene
regulation process. The siRNA therapy has been facing continuous barriers in the delivery
process to the tissues and cells of interest. The ultimate goal of this study was to use siRNA
therapy that showed efficacy in septic animals. We formulated an anti-TNF-α siRNA into a HA
polymer system with use of charge and hydrophobic interaction. The formulation showed
efficacious in macrophage cell uptake and TNF-α silencing. The TNF-α silencing showed to be associated with a cytokine flux reduction downstream of inflammation process. We believe that the significant survival benefit is a result of the “cytokine storm” silencing on the TNF-α signaling pathway. More importantly, we found that the local modulation of gene expression, results in systemic downregulation of the cytokine pool, which it is known to form during inflammation. The correlation between in vitro and in vivo studies indicates that a nanosized formulation is a potent approach for siRNA delivery, as well as indicated that the siRNA therapy has the potential to apply for sepsis therapy in clinic targeting TNF-α, provided that this target is moderately modulated. We would like to emphasize that the siRNA therapy might be of paramount success in those septic subject subpopulation where a pathogen is not present. However, we would also recommend that there is a lot that can be done on a combined therapy with antibiotics where pathogen is present. As it might be expected the results from this work, emphasize one more time, the need for an early diagnosis of sepsis syndrome, targeting of a powerful mediator as TNF-α, as well as the importance of timely synchronization of RNAi with cytokine storm pathway.
Appendix 1

1. Cytokine profile was assessed with use of Milliplex Map Kit. Below are the standard curves built by the software using logistic-5PL type of regression. The standard solutions were prepared according to the instructions and the curve was built based in fluorescence intensity measured by the instrument. The two controls provided with the kit were within the acceptance ranges as shown on table A-1.

![Graphs showing fluorescence intensity vs. concentration for IFN-\(\gamma\) and TNF-\(\alpha\)]

**Table A.1 Values of controls measured during the assay along with the expected ranges**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>IFN-(\gamma)</th>
<th>Range</th>
<th>TNF-(\alpha)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>122.49</td>
<td>(101-209)</td>
<td>111.47</td>
<td>(100-208)</td>
</tr>
<tr>
<td>Control 2</td>
<td>560.82</td>
<td>(595-1236)</td>
<td>679.16</td>
<td>(601-1344)</td>
</tr>
</tbody>
</table>

2. Quantities of RNA measured in cells and tissues after naked siRNA administration

In the tables below it is shown a comparison of active RNA strand quantities found in cells and tissues when the naked siRNA was injected, versus when the siRNA nanoparticle was injected.
As shown in Table A-2, the amount of active RNA, injected as naked siRNA, taken up by cells is 100 times less at its peak at 24 hours, than the amount that is delivered by the nanoparticle which is notably much higher for all the time points.

Table A-3. Amount of active RNA taken out by the liver from the naked siRNA injection, versus the amount of active RNA taken out by the liver injected with nanoparticle encapsulated siRNA

Table A-4. Amount of active RNA taken out by the spleen from the naked siRNA injection, versus the amount of active RNA taken out by the spleen injected with nanoparticle encapsulated siRNA

Table A-4. Concentration of the active RNA in plasma after naked siRNA injection, versus the concentration of active RNA in plasma after injecting nanoparticle encapsulated siRNA
As shown by these results, the formulation of the siRNA into a nanoparticle promotes the delivery into the peritoneal cells which are our cells of interest. The naked siRNA is measurable in cells only after 24 hours, and most probably at concentrations that do not promote silencing effect.

Table A-5. Concentration of the active RNA in kidney after naked siRNA injection, versus the concentration of active RNA in kidney after injecting nanoparticle encapsulated siRNA

<table>
<thead>
<tr>
<th>Time point (Hours)</th>
<th>Naked siRNA (ng/g kidney tissue)</th>
<th>Nanoparticle siRNA (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4.68</td>
<td>0.27</td>
</tr>
<tr>
<td>4</td>
<td>0.63</td>
<td>0.36</td>
</tr>
<tr>
<td>24</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>48</td>
<td>0.04</td>
<td>0.46</td>
</tr>
</tbody>
</table>

As shown by these results, the formulation of the siRNA into a nanoparticle promotes the delivery into the peritoneal cells which are our cells of interest. The naked siRNA is measurable in cells only after 24 hours, and most probably at concentrations that do not promote silencing effect.
References


