



**POLITECNICO
DI TORINO**

this:

POLITECNICO DI TORINO

Master Degree course in Biomedical Engineering

Master Degree Thesis

Transferrin Receptor-Targeted siRNA Delivery Using Functionalized PBAE Micelleplexes for Immune Cell Modulation

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Acknowledgements

Abbreviations

siRNA Small interfering RNA

pBAE Poly(β -Amino Ester)

P(SpOABAE) Poly(spermine oleylamine β -amino ester)

pSar Polysarcosine

PS LL14 Polysarcosine Lau–Ser(Lau)₁₄–NH₂

PS OO14 Polysarcosine Ole–Ser(Ole)–Sar₁₄–NH₂

PS LL28 Polysarcosine Lau–Ser(Lau)₂₈–NH₂

PS OO28 Polysarcosine Ole–L–Ser(Ole)–Sar₂₈–NH₂

PS P7 Polysarcosine Cap–Ser(Pal)–Sar₇–NH₂

LV Low viscosity

MV Medium viscosity

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

eGFP Enhanced green fluorescent protein

AF488 siRNA AlexaFluor488-labeled siRNA

Abstract

Advanced RNA-based immunotherapies have been widely investigated in recent years, due to their ability to regulate immune system response via RNA interference. Specifically, the use of siRNA to selectively modulate gene expression in activated immune cells represents a promising strategy; however, several challenges remain. Targeted siRNA delivery to activated immune cells is the key to obtain sequence-specific gene silencing, but since nucleic acids in their free form are rapidly degraded by nucleases present in extracellular matrices, have poor pharmacokinetics and hardly pass cellular membranes, carrier systems are required. Suitable carriers that have been extensively investigated are cationic lipids and cationic polymers due to their ability to encapsulate negatively charged nucleic acids through electrostatic interaction. In particular, polyplexes are interpolyelectrolyte complexes which are spontaneously formed through the electrostatic condensation between nucleic acid and a cationic polymer. Poly(β -Amino Ester)s (pBAEs) have emerged as attractive nucleic acid delivery materials owing to their biodegradability, structural tunability, and ability to efficiently complex and deliver nucleic acids. Nevertheless, pBAE-based carriers may still display dose- and formulation-dependent cytotoxicity. In this work, the polyplexes are based on amphiphilic poly(β -amino ester)s that self-assemble into micellar structures prior to siRNA complexation; therefore, the resulting complexes are referred to as micelleplexes. The objective is to optimize these formulations by shielding the pBAE micelleplexes to reduce cytotoxicity, enhance micelleplex stability, and maintain therapeutic efficacy. Herein, the following polymers were evaluated as shielding agents: alginate, poly- γ -glutamic acid, PEG-lipids, and polysarcosines. The coated micelleplexes were systematically evaluated in terms of size, ζ -potential, siRNA encapsulation and release efficiency. To have a broad physicochemical characterization, stability in physiologically relevant media was assessed by analyzing size and ζ -potential invariance in Phosphate-Buffered Saline (PBS), Fetal Bovine Serum (FBS), and Simulated Epithelial Lung Fluid (SELF). The cell culture work then allowed for the evaluation of the coated micelleplexes delivery efficiency by analyzing eGFP gene knockdown, as well as cytotoxicity in JE6.1-NF- κ B::eGFP cells. The transferrin receptor (TfR1/CD71), highly expressed on activated T cells, enables receptor-mediated uptake, making transferrin functionalization of micelleplexes a promising strategy to enhance cell specificity and uptake efficiency. pBAE micelleplexes were then transferrin-conjugated and characterized according to the same physicochemical and biological evaluation framework used for all previously tested formulations. Finally, the effectiveness of transferrin functionalization was evaluated by comparing micelleplex internalization in activated and non-activated CD4⁺ T cells.

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Chapter 1

Introduction

1.1 siRNA-based immunotherapies

RNA interference (RNAi) is a regulatory mechanism of eukaryotic cells to control gene expression [1]. RNAi offers the potential to specifically modulate the expression of any gene with a known sequence, enabling therapeutic downregulation of disease-associated genes [2]. Beyond this, it can be applied to conditions characterized by immune dysregulation, where it allows precise modulation of pathogenic immune pathways [3]. RNAi is an endogenous process for post-transcriptional gene silencing that is induced by double-stranded RNA (dsRNA), which includes endogenous microRNA (miRNA) and synthetic small interfering RNA (siRNA) [4]. Therefore, siRNA can be introduced directly into the cells or produced from long dsRNA cutting by an endo-ribonuclease named Dicer, as depicted in Figure 1. Once siRNA is in the cytoplasm, small interfering RNA is recognized and incorporated into RISC, the RNA-induced silencing complex, that separates the double-stranded siRNA by releasing the passenger strand. The now single-stranded guide RNA, still bound to the RISC complex, activates and directs the target recognition with the sequence-complementary messenger RNA (mRNA), which is consequently degraded or inactivated thus aborting target protein translation [5]. However, siRNA features some unfavorable physiological properties, including high susceptibility to serum nucleases and intrinsic physicochemical characteristics as negative charge, large size and hydrophilicity that collectively hinder efficient cellular uptake [6]. Furthermore, upon cellular uptake, siRNA may undergo endosomal trapping and subsequent lysosomal destruction [7]. Altogether, these factors result in decreased transfection efficacy and make efficient and reliable siRNA delivery the main bottleneck for therapeutic applications [8] [9]. To overcome these barriers, siRNA nanodelivery systems, including lipid-based siRNA nanocarriers, polymeric-based, inorganic material-based, and hybrid-based delivery systems, provide siRNA protection and enable better control over the delivery process. Despite the advantages, the lack of stability and the toxicity of NMs in the body environment pose a great challenge to in vivo applications [10].

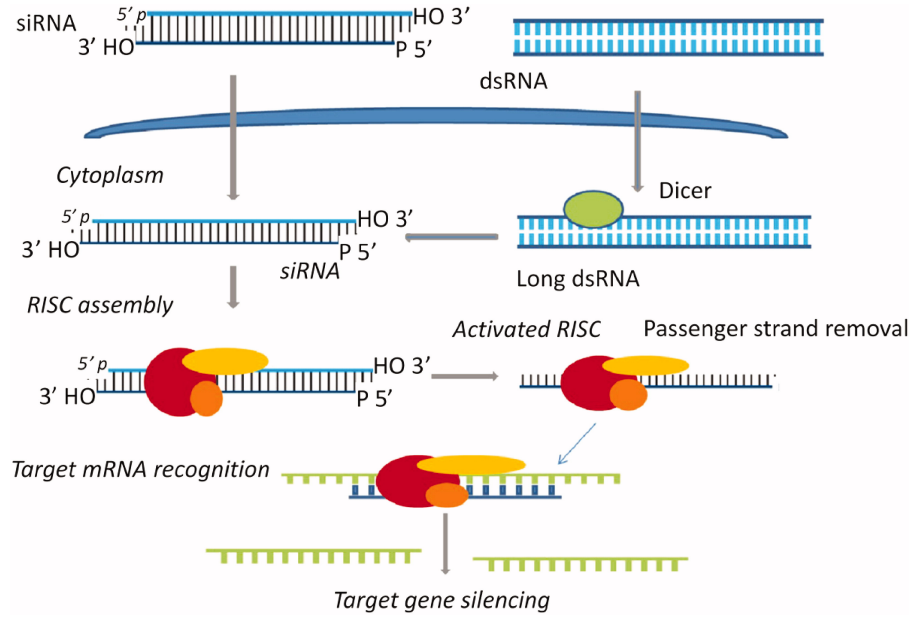


Figure 1: Mechanism of RNA interference (RNAi). Long double-stranded RNA (dsRNA) is cleaved by Dicer into siRNA in the cytoplasm, or siRNA can be directly introduced into the cell. The siRNA is then incorporated into the RISC complex, where the passenger strand is degraded while the guide strand directs the complex to complementary mRNA. This interaction leads to target mRNA degradation and subsequent gene silencing [5].

1.2 Polymer-based delivery system for siRNA delivery

Polymers are good candidates as nanodelivery system materials. Their advantage arises from their easy manipulation in terms of charge, degradability, and molecular weight, all of which influence how polymers deliver RNA into cells [11]. One frequently used polymer is poly(lactic- co- glycolic acid) (PLGA). At neutral pH, PLGA does not have the positive charge required to complex the anionic RNA phosphodiester backbone. Therefore, to be used as an RNA delivery system, it must be modified by the addition of cationic functional groups [12]. Polymers that contain amine groups that can be protonated, such as polyethylenimine (PEI) and poly(L-lysine) (PLL), can inherently complex with RNA via electrostatic interactions, forming polyplexes [13]. However, unmodified PEI and PLL are not always well tolerated, and PEI transfection capability and toxicity increase with molecular weight [14]. Thus, for example, PEI and PLL could be chemically modified by PEG grafting to improve in vivo efficacy and tolerability [15].

1.2.1 Poly (β -amino ester)s (PBAEs)

Another cationic polymer class is the poly(β - amino ester)s (PBAEs), which are synthesized by conjugating amine monomers to diacrylates. These polymers, which were designed to have improved biodegradation and cytotoxicity relative to PEI and PLL, contain cationic amines as well as biodegradable ester bonds. Early research used Michael

addition chemistry to synthesize hundreds of chemically distinct PBAEs and then evaluated how these nanoparticles delivered DNA and RNA in cell culture [16]. This approach enables the understanding of how structural differences affect DNA delivery efficiency, thereby providing design rules for subsequent pBAEs. Initial findings suggested that effective polymers were almost always hydrophobic, had monoalcohol or dialcohol side groups, and had linear bis (secondary) amines [14]. The pBAE used in this study is functionalized with spermine and oleylamine at a defined 32:68 molar ratio (Figure 2). Spermine is a polycationic moiety, raising the overall positive charge of the polymer. This effect improves electrostatic complexation with siRNA and promotes interaction with the cell surface, leading to more efficient micelleplex formation and internalization. Hydrophobic oleylamine chains interact with endosomal membrane lipids, facilitating membrane destabilization and subsequent micelleplex endosomal escape [17]. Despite these promising properties, pBAE-based micelleplexes may exhibit limited colloidal stability in biological environments, formulation-dependent cytotoxicity, and may trigger inflammatory responses [18] [19].

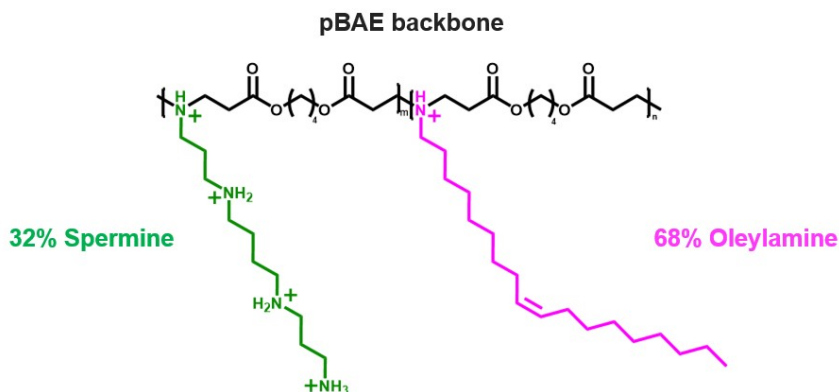


Figure 2: Chemical structure of the pBAE functionalized with spermine (32 mol%) and oleylamine (68 mol%). Spermine provides a cationic moiety to the polymer, whereas oleylamine imparts hydrophobicity.

1.2.2 Coating polymers

The strategy adopted in this work relies on the shielding of P(SpOABAE) micelleplexes using different polymers, with the aim of addressing the key limitations of the uncoated formulation and improving overall performance. When classified according to their coating mechanism, the first category comprises alginate and PGA (poly- γ -glutamic acid), whose negative charge at physiological pH enables electrostatic interactions with P(SpOABAE) micelleplexes. Alginate (Figure 3a) is a natural polysaccharide composed of 1,4-linked β -D-mannuronic acid (M) and 1,4 α -L-guluronic acid (G) residues [20], whose carboxyl groups are deprotonated at physiological pH. Poly- γ -glutamic acid (Figure 3b) is a synthetic polypeptide made of D- and L-glutamic acid units [21], each of which contains a carboxyl group, also deprotonated at physiological pH. Coating with anionic

polymers may help to neutralize or slightly reverse the intrinsic positive surface charge of P(SpOABAE), thereby potentially reducing the toxicity of the micelleplexes. For each polymer, two different molecular weights have been investigated, under the hypothesis that the molecular weight of the shielding polymer could influence its interaction with the P(SpOABAE) and thereby affect coating formation and coating properties such as stability, density, and functionality.

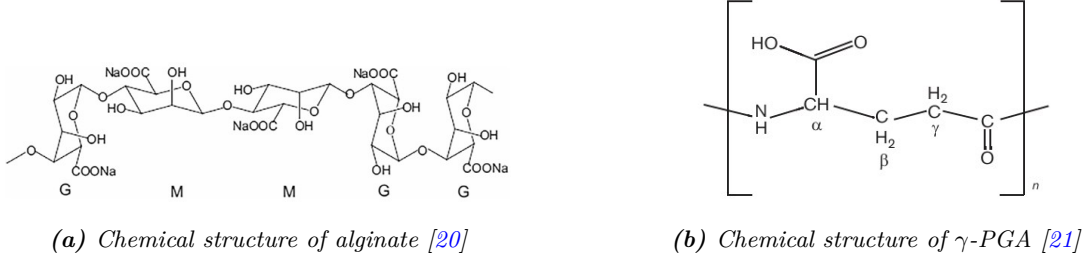


Figure 3: Coating polymers electrostatically interacting with pBAE

Moving on to the next class of coating polymers, three amphiphilic molecules have been investigated, each consisting of a PEG chain covalently attached to a lipid tail (Figure 4). The lipid portion (two myristoyl chains in DMG-PEG (Figure 4a) and DMPE-PEG (Figure 4b), and two stearyl chains in DSPE-PEG (Figure 4c)) preferentially inserts into the hydrophobic domains of P(SpOABAE) (e.g., oleylamine chains). This non-covalent insertion anchors the PEG chains to the polyplex surface, forming an external steric brush. As previously discussed, it is hypothesized that differences in lipid chain length may influence their interaction with the pBAE.

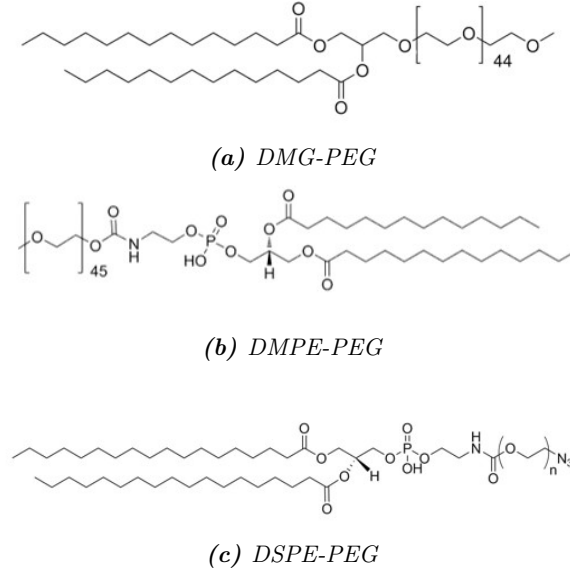


Figure 4: Coating polymers interacting with pBAE via hydrophobic anchoring

The final class comprises five different types of polysarcosines. Polysarcosines are hydrophilic, non-ionic polypeptoids consisting of repeat units of N methyl glycine. Because it is hydrophilic, biocompatible, biodegradable and shows no reported cytotoxicity or immunogenicity, pSar is promoted as a “stealth” alternative to poly(ethylene glycol) (PEG) for drug delivery, protein conjugation and nanoparticle coronas. The pSar blocks form a hydrophilic, PEG like surface that shields the positively charged pBAE core, reducing colloidal aggregation and PEG associated reactogenicity while preserving the pBAE ability to complex RNA [22]. pSars can interact with the P(SpOABAE) through dipole-dipole interactions between the ester carbonyl groups of the pBAE and the amide carbonyl groups of polysarcosine, along with additional hydrogen bonding with pSar carbonyl groups acting as hydrogen bond acceptors. Moreover, potential hydrophobic interactions with oleylamine chains may occur, overall resulting in a non-covalent surface adsorption of polysarcosine onto P(SpOABAE) micelleplexes. All of the polysarcosines involved in this study share a common backbone, while differing in chain length and the nature of their terminal hydrophobic moieties (Lau, Ole, Pal), as shown in Figure 5 . For the first two variants, two distinct chain lengths were considered (Sar14 and Sar28), whereas the Pal polysarcosine has the shortest chain length (Sar7). This additionally allows the evaluation of the effect of these structural variations on coating formation, following the same rationale as for the other polymer classes. Three distinct micelleplex formulations were tested, each characterized by different P(SpOABAE)-to-coating polymer molar ratios: 1:0.005 (0.5mol%), 1:0.025 (2.5mol%), and 1:0.05 (5mol%). In particular, the spermine-to-coating polymer molar ratio was considered for Alginate- and PGA-coated formulations, as these polymers directly interact with the cationic moieties of P(SpOABAE), whereas the oleylamine-to-coating polymer molar ratio was adopted for pSars- and PEG-coated formulations, given the preferential interaction of pSars and PEGs with the hydrophobic oleylamine chains.

1.3 Physicochemical characterization techniques applied to the formulations

The physicochemical characterization of micelleplexes plays a pivotal role in determining their transfection efficiency, given that basic features such as particle size and surface charge critically influence their interactions with biological environments and cells; therefore, a systematic evaluation starting from these primary characteristics is essential to assess whether a formulation represents a suitable candidate for efficient gene delivery. Considering micelleplexes size, a cutoff value around 100 nm is generally considered appropriate, as larger nanoparticles are more prone to aggregation and are more susceptible to rapid clearance in vivo. It is also considered a favorable size range for cellular uptake, with particles below 150 nm having been reported to be required for efficient endocytosis in lung cells. The size of bulk micelleplexes is measured by Dynamic Light Scattering (DLS), a technique used to determine the size distribution profile of small particles in solution or suspension. The same analysis also provides the polydispersity index (PDI), with values below 0,3 indicating a monodisperse population, which in turn reflects good

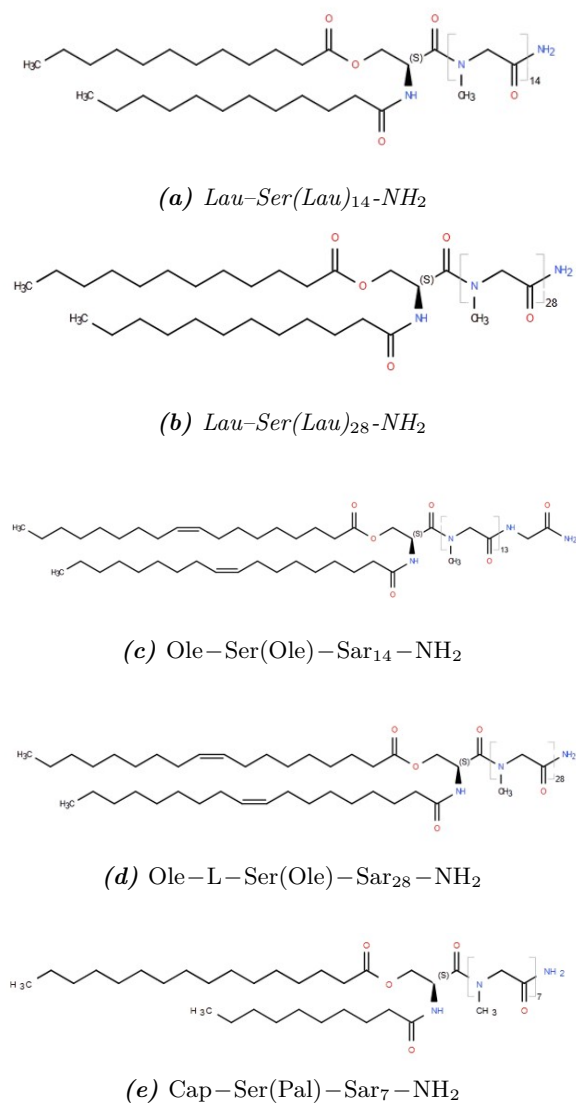


Figure 5: Coating polymers interacting with pBAE via surface adsorption

colloidal stability, minimal aggregation, and more reproducible and reliable results. Electrophoretic light scattering (ELS) is used to determine the zeta potential of particles in solution or suspension and thereby assess the surface charge of micelleplexes. A near-neutral zeta potential was targeted, as the coating polymers are also designed to shield the intrinsic positive charge of P(SpOABAE) micelleplexes, which may contribute to cytotoxicity and non-specific cellular uptake.

1.3.1 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is based on scattering of light by particles in a colloidal dispersion. The equipment focuses a beam of laser light into the nanoparticle solution, and a photon detector measures the intensity of the scattered light. The continuously Brownian movement of the particles within dispersion cause constructive and destructive interferences and hence, the intensity of scattered light fluctuates over time. The fluctuation of intensity in scattered light is correlated against short decay intervals (τ) and the intensity ACF (autocorrelation function) is obtained through the following mono-exponential equation for samples with purely monodisperse particles:

$$G(\tau) = 1 + b e^{-2D_t q^2 \tau} \quad (1.1)$$

Here, b is a constant dependent upon the instrument and settings of optics, D_t is the translational diffusion coefficient and q is the scattering vector [23].

According to the particles diffusing speed, correlation decay time constants derived from the exponential auto correlation function (ACF) are different, which is used to calculate the diffusion coefficient (D_t). This can be subsequently used to calculate the hydrodynamic radius r_h of a corresponding spherical particle using the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta r_h} \quad (1.2)$$

k_B Boltzmann constant (1.380648×10^{-23} J K⁻¹)

T Temperature

η Viscosity

D Diffusion constant

r_h Hydrodynamic radius

This relation depends on both the viscosity of the sample solution and the temperature at which the scattered light is collected.

1.3.2 Electrophoretic Light Scattering (ELS)

Electrophoretic light scattering (ELS) is a technique used to determine the electrophoretic mobility of particles dispersed in a colloidal system. In this method, a laser beam is directed through the nanoparticle dispersion while an external electric field is applied across the sample. Charged particles move toward the electrode of opposite charge with a velocity proportional to their electrophoretic mobility. The motion of the particles induces a frequency shift in the scattered light due to the Doppler effect. This shift is detected by a photodetector and analyzed to determine the velocity of the particles. The frequency change is directly proportional to the electrophoretic velocity, which allows the calculation of the electrophoretic mobility (μ_e).

The electrophoretic mobility is defined as the ratio between the particle velocity(v) and the applied electric field strength(E): $\mu_e = \frac{v}{E}$

From the electrophoretic mobility, the ζ -potential can be calculated using the Henry Equation [23]:

$$\mu_e = \frac{2\varepsilon_r\varepsilon_0\zeta f(Ka)}{3\eta} \quad (1.3)$$

ε_r Dielectric constant

ε_0 Permittivity of vacuum

ζ Zeta potential

$f(Ka)$ Henry's function

η Viscosity

Similarly to DLS, this relation depends on the temperature at which the measurement is performed and the viscosity and ionic strength of the sample solution, all of which influence the electrical double layer surrounding the particles and, therefore, their overall electrophoretic behavior.

1.4 Targeted delivery via Transferrin-conjugated micelleplexes

Immunotherapies aim to modulate the immune response in order to restore immune homeostasis or enhance protective immunity in pathological conditions. Among immune cell populations, T lymphocytes play a central role in orchestrating adaptive immune responses and therefore represent attractive therapeutic targets [24]. In pathological conditions characterized by immune activation, T cells upregulate the expression of transferrin receptor 1 (TfR1, CD71) [25]. Consequently, the conjugation of transferrin to nanocarriers has emerged as a promising strategy to promote preferential uptake by activated T cells while minimizing off-target interactions [26] [27]. In this context, the functionalization of micelleplexes with transferrin is designed to exploit the overexpression of CD71 on activated T cells to achieve targeted delivery of siRNA, potentially improving the efficacy of immunomodulatory interventions while reducing systemic exposure [28] [29].

1.5 Ambition

The aim of this work is to optimize the formulation of P(SpOABAE) micelleplexes for targeted siRNA delivery, in order to reduce cytotoxicity, improve micelleplex stability, while maintaining or enhancing transfection efficacy. To achieve this objective, different coated formulations were developed and systematically evaluated based on their physicochemical properties, stability in biologically relevant media, and biological performance. This initial screening allows for the partial exclusion of formulations that do not adequately meet

the predefined selection criteria and performance requirements. For biological testing of the selected formulations, a range of siRNA doses (2.5?100 pmol) was evaluated in order to identify the critical dose that ensures effective gene knockdown with minimal toxicity. Gene silencing efficiency was assessed by eGFP gene knockdown measured by flow cytometry, while cytotoxicity was measured by LDH release assay in activated Jurkat NF- κ B reporter T cells. Subsequently, an erythrocyte lysis assay was performed to assess the hemocompatibility of each formulation across the range of siRNA doses considered. These evaluations were designed to assess the balance between gene silencing activity, cytocompatibility, and hemocompatibility, thereby supporting the selection of formulations most suitable for further in vivo studies.

The second objective of this work is to achieve a targeted siRNA delivery through the conjugation of transferrin to optimized siRNA micelleplexes. This approach aims to improve selective uptake in T cells while maintaining low toxicity profiles and gene silencing capability. The impact of transferrin conjugation on cytocompatibility was investigated using primary human PBMCs. A luminescent cell viability assay was employed to evaluate alterations in cellular metabolic activity that may not be detected by membrane integrity-based assays. Gene silencing efficiency was evaluated under the same experimental conditions used for the unconjugated counterparts, to determine whether transferrin functionalization affected their performance. Finally, the targeting ability conferred by transferrin conjugation was assessed by analyzing the preferential uptake of micelleplexes in activated CD4+ T cells compared to non-activated ones. Overall, this work seeks to contribute to the development of stable and biocompatible polymeric siRNA delivery systems with optimized physicochemical properties and selective targeting of immune cells, thereby supporting their potential application in immunotherapies.

Chapter 2

Materials and Methods

2.1 Materials

For micelleplexes preparation, the P(SpOABAE) was obtained by The Merkel synthesis Lab, while HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was purchased from Sigma-Aldrich (Taufkirchen, Germany). Concerning the coated formulations, alginates were purchased from Sigma-Aldrich (Taufkirchen, Germany), PGAs from ALAMANDA Polymers (Huntsville, AL, USA), DMPE-PEG from Lipoid (Ludwigshafen, Germany), while DMG-PEG and DSPE-PEG from Avanti Polar Lipids (Alabaster, AL, USA). All polysarcosines were obtained from the Merkel synthesis Lab. For micelleplexes characterization, SYBR Gold Dye was purchased from Life Technologies (Carlsbad, USA), while Heparin from Thermo Fisher Scientific (Waltham, USA) and Triton X-100 from Sigma-Aldrich, Taufkirchen, Germany. For cell culture, RPMI-1640 Medium, fetal bovine serum (FBS), Penicillin-Streptomycin solution, dimethyl sulfoxide (DMSO) and Dulbecco's Phosphate Buffered Saline (PBS) were purchased from Sigma-Aldrich (Taufkirchen, Germany). CytoTox 96[®] Non-Radioactive Cytotoxicity Assay and CellTiter-Glo[®] Luminescent Cell Viability Assay are products of Promega Corporation (Madison, WI, USA). AlexaFluor488-labeled siRNA and Lipofectamine 2000 for the cellular uptake assay were purchased from (Thermo Fisher Scientific, Schwerte, Germany).

2.2 Micelleplexes preparation

The polymers stock solutions and the siRNA stock solution were diluted in 10 mM freshly filtered HEPES buffer (pH 5.4). In 1.5 mL microcentrifuge tubes the desired volume of diluted polymer solution was added to an equal volume of diluted siRNA solution, followed by immediate mixing by pipetting. After 30 minutes incubation at room temperature, the micelleplex formulation is stable. A standard siRNA dose of 25 pmol per sample was used, unless otherwise specified. The N/P ratio is defined as the molar ratio between the polymer amine groups (N) and the siRNA phosphate groups (P). The amount of the polymers required to achieve various N/P ratios with a specific amount of siRNA was determined using the following equation [30]:

$$m(\text{polymer in pg}) = n_{\text{siRNA}}(\text{pmol}) \cdot M_{\text{protonable unit}} \left(\frac{\text{g}}{\text{mol}} \right) \cdot \text{N/P} \cdot \text{number of nucleotides}_{\text{siRNA}} \quad (2.1)$$

where m is the mass of the polymers is needed, and n is the amount of siRNA used per well. The protonable unit M of each polymer is calculated by dividing its molar mass by the number of protonatable amines in each polymer. The number of nucleotides of 25/27mer siRNA is 52. Coated formulations were prepared using stock solutions of the coating candidate polymers. Polysarcosines and PEGs were dissolved in absolute ethanol ($\geq 99.5\%$, molecular biology grade), while Alginates and PGAs were dissolved in 10 mM freshly filtered HEPES buffer (pH 7.4).

In both approaches, the total volume of the polymer solution was kept constant. For coated formulations, the addition of the coating polymer to achieve the desired P(SpOABAE)-to-coating polymer molar ratios (1:0.005, 1:0.025, and 1:0.05) was compensated by a proportional reduction in the solvent volume.

2.3 Size and Zeta (ζ)-Potential analysis of micelleplexes by DLS and ELS

Dynamic Light Scattering (DLS) was used to determine sizes and polydispersity index (PDI) of the micelleplexes, starting from the uncoated formulations with different N/P ratio. Measurements were performed in triplicate using a Zetasizer Nano ZS (Malvern Instruments) by setting 173° backscatter angle and 6 sub-runs per measurement. A volume of 100 μL of each formulation was prepared as described above and transferred into a disposable cuvette (Malvern Instruments, Malvern, UK) for analysis with the following settings: 30 s of equilibration time, temperature 25°C , refractive index 1.330, and viscosity 0.891 mPa.s. To determine the ζ -potential, the micelleplex solution was diluted with 700 μL of HEPES buffer (pH 7.4) and transferred into a folded capillary cell (DTS1070, Malvern Instruments Inc.) prior to measurement. The Zeta (ζ)-potential was measured by Electrophoretic Light Scattering (ELS). Each sample was analyzed in triplicate, with 15 sub-runs of 10 s each, and ζ -potential was calculated by Smoluchowski approximation of the Henry equation. Measurement settings were identical to those used for size determination. All results (size, PDI, and ζ -potential) were reported as mean $\pm$ SD ($n = 3$) out of these measurements. The same Zetasizer measurements were subsequently performed on all coated formulations.

2.4 siRNA encapsulation and release efficiency by SYBR Gold Assay

The ability of micelleplexes to encapsulate and release siRNA was evaluated using the SYBR Gold assay, a fluorescence-based method for nucleic acid quantification. This

method relies on the binding of the SYBR Gold dye to nucleic acids, resulting in enhanced dye fluorescence and thus increased sensitivity for siRNA detection. Encapsulated siRNA within nanoparticles remains inaccessible to the dye until it is released from the nanoparticle structure. Fluorescence is then measured by spectrophotometry and is directly proportional to the amount of siRNA released from the micelleplexes. As for the other previous tests, uncoated formulations at different N/P ratios were analyzed first, followed then by each coated formulation. Micelleplexes were prepared as described in Section 2.2 and encapsulation efficiency was determined following the protocol described in [30]. 20 μL of each polyplex sample was transferred in triplicate to a 96-well black microplate. The same amount of the “free siRNA” sample (N/P = 0) was added in triplicate to another section of wells of the same microplate, as a 100% release control. Lastly, 20 μL of formulation buffer was then transferred to a separate set of wells, designated as “blank”. Release efficiency was evaluated following the procedure described in [30]. First, Tris-EDTA (TE) buffer solution 100X (Sigma-Aldrich, Taufkirchen, Germany) was diluted in RNase-free water to obtain the 1X TE buffer. 2% Triton X-100 solution was then prepared by diluting an appropriate volume of Triton X-100 in TE buffer. The heparin solution was prepared by dissolving heparin sodium salt (Thermo Fisher Scientific, Waltham, MA, USA) in RNase-free water to a final concentration of 2000 U/mL. Next, 10 μL of each micelleplex sample was transferred in triplicate to a 96-well black microplate. The same amount of the “free siRNA” sample and formulation buffer were added in triplicate to separate sets of wells, serving as 100% release control and “blank”, respectively. Subsequently, 10 μL of 2% Triton X-100 solution was added to each sample, followed by 2 μL of heparin solution. The plate was then incubated for 1 h at 37 °C under shaking at 300 rpm.

Once incubation was complete, 3 μL of 4X SYBR Gold working solution was added to each sample and the plate was then wrapped with aluminum foil and shaken for 5 minutes at room temperature on the plate shaker set at 300 rpm. Thereafter, fluorescence was measured using the same excitation and emission wavelengths as described before. For both encapsulation and release experiments, the amount of free siRNA in corresponding samples was quantified using the following formula:

$$\text{Free siRNA [\%]} = \frac{\text{FI Sample [Polyplex/Micelleplex]} - \text{FI Sample [Blank]}}{\text{FI Sample [Free siRNA]} - \text{FI Sample [Blank]}} \times 100 \quad (2.2)$$

The N/P ratio of 0 corresponds to the sample “free siRNA” and is mathematically set to 100% free siRNA (FI = Fluorescence Intensity [AU]).

2.5 Colloidal stability of micelleplexes in PBS

The uncoated formulation and all coated formulations were assessed for colloidal stability in PBS over time. Micelleplex solutions (50 μL) were formed as described above (Section 2.2) and diluted with 87.5 μL of PBS, following a protocol adapted from [31]. Subsequently, size was measured at different time points (0, 1, 3, and 24 h) by DLS using the same settings as described in Section 2.3.

2.6 Stability of micelleplexes in serum and Simulated Epithelial Lung Fluid (SELF)

The uncoated formulations and all coated formulations were assessed for stability in two biologically relevant fluids, namely Fetal Bovine Serum (FBS) and Simulated Epithelial Lung Fluid. First, SELF was prepared following a protocol adapted from [32]. Briefly, the chemicals listed in Table 1 (A) and (B) were dissolved in deionized water in separate glass reagent bottles, yielding equal volumes of the inorganic and organic solutions. The two phases were then combined in a new glass reagent bottle, which contained the solids specified in Table 1 (C), and the resulting fluid was mixed thoroughly.

Table 1: Simulated epithelial lung fluid formulation. Data adapted from [32].

Reagent	Weight (mg) in 500 mL
<i>(A) Inorganic phase reagents</i>	
NaCl	6020
CaCl ₂	256
Na ₂ HPO ₄	150
NaHCO ₃	2700
KCl	298
MgCl ₂	200
Na ₂ SO ₄	72
<i>(B) Organic phase reagents</i>	
Ascorbic acid	18
Uric acid	16
Glutathione	30
<i>(C) Additional reagents</i>	
Albumin	260
Cysteine	122
DPPC	100
Glycine	376
Mucin	500

Finally, the pH of the solution was measured and, if necessary, adjusted to 7.4 ± 0.2 by addition of an appropriate amount of HCl. Micelleplex solutions (100 μ L) were prepared as described above (Section 2.2), using a concentration of 100 pmol siRNA / 50 μ L. Subsequently, 25 μ L of FBS were added to one 50 μ L aliquot of the micelleplex solution, while 12,5 μ L of SELF were added to a second 50 μ L aliquot. Background controls were prepared at the same total volume, consisting of micelleplex formulation buffer combined with serum and SELF in the same proportions as the corresponding samples, but in the absence of micelleplexes. These controls were used as references for comparison. The samples were then incubated for 2 h at 37 °C under continuous shaking at 300 rpm, following a protocol adapted from [31]. After incubation, each 50 μ L sample was diluted with 50 μ L of HEPES buffer (pH 7.4) prior to size and ζ -potential measurements. DLS settings were identical to those described in Section 2.3, except for an increase in equilibration time to 60 seconds.

2.7 Cells and cell Culture

The JE6.1-NF- κ B::eGFP reporter cell line, derived from the human Jurkat E6.1 T cell line, was cultured in RPMI-1640 medium supplemented with 10% FBS. Cells were maintained in suspension culture at 37 °C in a humidified atmosphere with 5% CO₂. The reporter construct encodes eGFP under the control of NF- κ B response elements, allowing the monitoring of NF- κ B activation. Cells were maintained in T75 vented tissue culture flasks and passaged three times per week to a density of 3×10^5 cells/mL.

2.8 *In Vitro* eGFP knockdown by flow cytometry

An enhanced green fluorescent protein reporter gene (eGFP) silencing experiment was conducted to evaluate whether micelleplexes can effectively knockdown protein levels in cells. To this end, JE6.1-NF- κ B::eGFP cells were stimulated with PMA (phorbol 12-myristate 13-acetate) and ionomycin prior to treatment, in order to induce NF- κ B activation and reporter gene expression, thereby mimicking an inflamed microenvironment representative of the target conditions for polyplex delivery to activated T cells. PMA stock and ionomycin stock solutions were first diluted in PBS and then added to Penicillin/Streptomycin -supplemented culture medium to achieve final concentrations of 100 nM and 0.25 μ M per well, respectively. The required volume of the resulting medium (hereafter referred to as activated medium) was calculated assuming a total volume of 200 μ L per well, including samples and controls in triplicate. The “Activated Blank” consisted of triplicate wells with untreated cells receiving the same buffer used to deliver the test compounds (50 μ L of HEPES pH 5.4), while the “Non-Activated Blank” consisted of triplicate wells with untreated cells in non-activated medium receiving the same solvent. Subsequently, the required number of Jurkat cells (100 000 cells per well) was collected and resuspended in activated medium to reach a concentration of 500 000 cells/mL. Cells for the three “Non-Activated Blank” wells were instead resuspended in complete P/S-supplemented medium. Micelleplexes were prepared as previously described in Section 2.2, using increasing siRNA doses per sample (12.5, 25, 35, 50, 75, 100, and 200 pmol) to generate an initial dose-response profile. For each siRNA dose, two polyplex formulations were prepared: one containing a non-targeting control siRNA and the other containing a siRNA targeting eGFP, which was expected to induce gene knockdown in JE6.1-NF- κ B::eGFP cells. Upon seeding in flat-bottom 96-well plates, cells were transfected with 50 μ L of particles per well. Plates were incubated for 48 h at 37 °C and 5% CO₂. Cells were then collected and processed for flow cytometry. Plates were first centrifuged at 500 rpm for 5 minutes to pellet the cells. The supernatant was collected for the LDH release cytotoxicity assay, and cells were washed three times with 200 μ L of PBS, with centrifugation between washes. Cells were resuspended in 200 μ L of PBS supplemented with 2 mM EDTA and 0.5% BSA prior to flow cytometric analysis. The samples were examined using an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a 488 nm excitation laser and a 535 nm emission filter. All cells were gated according to their morphology using forward/sideward scattering, and each sample had 10.000 events.

Median fluorescence intensity (MFI) was obtained for each sample and normalized to the mean MFI of the “Activated blank” wells, according to the following equation:

$$\text{Normalized MFI (\%)} = 100 \times \frac{\text{MFI}_{\text{sample}}}{\text{MFI}_{\text{activated blank}}} \quad (2.3)$$

The “Activated Blank” was used as the reference maximum, as it represents the highest level of eGFP expression under activated conditions in the absence of treatment.

Results are expressed as eGFP reduction (%), calculated as $100 - \text{Normalized MFI}$, and reported as mean $\pm$ SD ($n = 3$).

2.9 LDH release cytotoxicity assay

All formulations tested for eGFP knockdown were also evaluated for cytotoxicity using the CytoTox 96® Non-Radioactive Cytotoxicity Assay. Triplicate wells containing untreated cells in activated medium were incubated in parallel with the knockdown samples for 48 h and served as the “Vehicle-Only Cells Control”. The “Maximum LDH Release Control” was prepared by adding 20 μL of 10X Lysis Solution per 100 μL of “Vehicle-Only Cells Control” at least 2 h prior to the addition of the CytoTox 96® Reagent. Subsequently, the supernatant was collected from each well following the 48 h incubation period required for eGFP knockdown and transferred (50 μL per sample, in triplicate) into a flat-bottom 96-well plate. Supernatant from the “Activated Blank” wells was included as the baseline cytotoxicity control for comparison with micelleplex-treated cells. A volume of 50 μL of CytoTox 96® Reagent was added to each well, and the plate was subsequently wrapped in aluminum foil and incubated at room temperature for 30 minutes. Upon completion of the incubation period, 50 μL of Stop Solution was added to each well, and the absorbance at 490 nm or 492 nm was measured within 1 h after Stop Solution addition using a plate reader (TECAN, Männedorf, Switzerland). Any large bubbles were carefully removed with a syringe needle prior to read-out. The cytotoxicity percentage associated with each tested formulation was calculated using the following formula:

$$\text{Percent cytotoxicity[\%]} = 100 \times \frac{\text{Experimental LDH Release } (OD_{490})}{\text{Maximum LDH Release } (OD_{490})} \quad (2.4)$$

Where “Maximum LDH Release” corresponds to the average absorbance value of the “Maximum LDH Release Control” wells.

2.10 Erythrocyte lysis assay

The hemolytic activity of micelleplexes was further investigated using a hemolysis assay. Micelleplex solutions (42 μL) were prepared at increasing siRNA doses, as described in Section 2.8. Human erythrocytes were isolated from human blood and red blood cells were washed in PBS until the supernatant was clear and colorless. Erythrocytes were then diluted in PBS to a final concentration of 5×10^8 cells/mL. A volume of 50 μL per well of the erythrocyte suspension was transferred into a flat-bottom 96-well plate. PBS (0% lysis) and 1% Triton X-100 in PBS (100% lysis) were added in triplicate as

negative and positive controls, respectively. 8 μL of 30% glucose solution was added to 42 μL of each micelleplex solution right before mixing it with the erythrocyte suspension in the well. This allowed the final glucose concentration to reach 5%, ensuring isotonic conditions. After sample addition, the plate was incubated for 30 minutes at 37 °C under constant shaking. After centrifugation at $850 \times g$, the supernatant was transferred to a new plate and analyzed for hemoglobin release at 541 nm. Experiments were carried out in triplicate, and hemolysis values [%] were calculated using the following formula:

$$\text{Percent hemolysis} = 100 \times \frac{\text{Experimental hemoglobin release} - \text{background}}{\text{Maximum hemoglobin release} - \text{background}} \quad (2.5)$$

Where “Maximum hemoglobin release” corresponds to the average hemoglobin release measured in the “100% lysis” control wells, while “background” corresponds to the average hemoglobin release of the “0% lysis” control wells. Results are expressed as mean $\pm$ SD for each sample.

2.11 Transferrin-conjugation of micelleplexes

For transferrin conjugation, a transferrin solution was first prepared by diluting an appropriate volume of the transferrin stock in 10 mM HEPES buffer (pH 7.4), to obtain a molar amount of transferrin equivalent to that of DSPE-PEG at a P(SpOABAE)-to-coating polymer molar ratio of 1:0.005 (0.5 mol%). The transferrin solution was then added to the DSPE-PEG Azide-coated micelleplex suspension at a 1:1 volume ratio. The resulting formulation was incubated for 2 h at room temperature to allow surface conjugation of transferrin.

2.12 CellTiter-Glo viability assay

Cell viability was assessed using the CellTiter-Glo[®] Luminescent Cell Viability Assay, which quantifies intracellular ATP as an indicator of metabolically active cells. Upon addition of the reagent, cells are lysed and ATP is released, driving a luciferase-mediated reaction that produces a luminescent signal proportional to the number of viable cells [33]. While hemolysis and LDH release assays provide information on membrane disruption and late-stage cytotoxicity, respectively, the CellTiter-Glo[®] assay enables the detection of early metabolic impairment by quantifying intracellular ATP levels. This allows the identification of sub-lethal effects that are not captured by membrane integrity-based assays [34–36]. Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats by Ficoll-Paque Plus density-gradient centrifugation. Cell density was subsequently assessed to ensure that measurements were performed within the linear range required for the CellTiter-Glo[®] Assay. Cells were seeded at 100,000 cells per well in 200 μL of RPMI-1640 medium supplemented with 10% FBS and P/S. Three medium-only control wells were included to determine background luminescence (“blank”). The plate was maintained in suspension culture at 37 °C in a humidified atmosphere with 5% CO₂ for 24 h. Subsequently, cells were transfected in triplicate with 50 μL of uncoated

nanoparticles, DSPE-PEG-coated nanoparticles, and transferrin-conjugated DSPE-PEG-coated nanoparticles. Micelleplexes were prepared as described in Section 2.2, and transferrin conjugation was performed as described in Section 2.11. Both conjugated and non-conjugated micelleplexes were then evaluated across a siRNA dose range of 6.25, 12.5, 25, 50, and 100 pmol. Three wells containing untreated cells served as “100% viability control”. After 24 h of incubation, 100 μ L of CellTiter-Glo[®] solution was added to each well. The plate was then shaken on an orbital shaker for 2 min to induce cell lysis. Before recording luminescence, the plate was incubated at room temperature for 10 min to allow stabilization of the luminescent signal. Cell viability was calculated by normalizing sample luminescence to untreated control cells after subtraction of background luminescence (“blank”), according to the following equation:

$$\text{Cell viability (\%)} = 100 \times \frac{\text{Sample luminescence} - \text{Background}}{\text{Maximum luminescence} - \text{Background}} \quad (2.6)$$

With Maximum luminescence corresponding to “100% viability control”. Results are expressed as mean $\pm$ SD (n = 3).

2.13 Transferrin-conjugated micelleplexes uptake in CD4⁺ T cells

To evaluate the selective uptake efficiency of transferrin-conjugated micelleplexes, an ex vivo uptake experiment was performed on CD4⁺ T cells isolated from PBMCs. CD4⁺ T cells were seeded at a density of 1×10^5 cells per well in 200 μ L of RPMI medium supplemented with 10% FBS and P/S. An equal number of wells was either left non-activated or activated using Dynabeads at a 1:1 bead-to-cell ratio, according to the manufacturer’s instructions. Upon activation, T cells upregulate the transferrin receptor (TfR1/CD71), thereby potentially enhancing the uptake of transferrin-conjugated micelleplexes. The plates were then incubated at 37 °C in a humidified atmosphere with 5% CO₂ for 24 h. Following this period, micelleplexes were prepared as described in Section 2.12 using a mixture of fluorescently labelled siRNA (AF488) and unlabelled siRNA at a fixed total dose of 50 pmol in 50 μ L. Activated and non-activated cells were transfected in triplicate with Tf-DSPE-PEG-pBAE- and DSPE-PEG-pBAE-coated micelleplexes. Control groups included untreated cells (“blank”), “free siRNA” as negative control, and Lipofectamine 2000 as a positive control for transfection efficiency. Cells were washed and resuspended in PBS as described in Section 2.8. The samples were then examined using an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a 488 nm excitation laser and a 535 nm emission filter. All cells were gated according to their morphology using forward/sideward scattering, and 10,000 events were recorded per sample. The percentage of AF488-positive cells was determined for both activated and non-activated CD4⁺ T cells, allowing comparison of nanoparticle uptake between the two conditions. Results are expressed as Cellular Uptake (%) and reported as mean $\pm$ SD (n=3).

Chapter 3

Results and Discussion

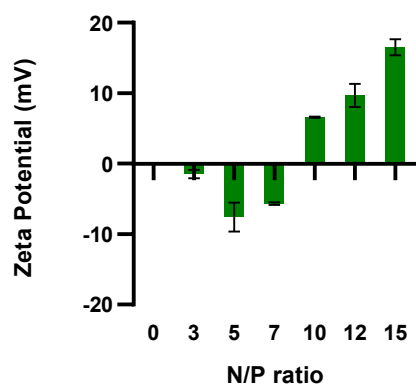
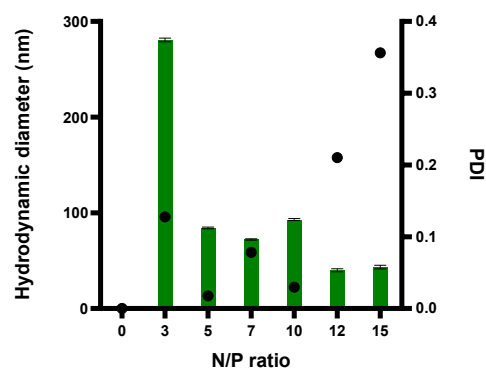
3.1 Physicochemical characterization of uncoated P(SpOABAE) micelleplexes

To enable a systematic evaluation of the different coating strategies, the uncoated formulation was first characterized, serving as a baseline for comparison with all subsequent results. Specifically, non-coated P(SpOABAE) micelleplexes prepared at different N/P ratios were analyzed to identify the optimal N/P value to be selected for further investigations. As shown in Figure 6a, zeta potential values remained close to neutrality for almost every formulation tested. This suggests that P(SpOABAE) positive charge was effectively shielded, potentially reducing charge-mediated cytotoxicity and non-specific cellular uptake. Formulations prepared at 5, 7 and 10 N/P ratios exhibited optimal hydrodynamic diameters below 100 nm (Figure 6b). This size range is favorable for efficient cellular uptake while limiting rapid clearance by the immune system, whereas larger nanoparticles are more prone to aggregation. In addition, low PDI values below 0.1 indicated monodisperse micelleplexes solutions, ensuring reproducibility, increased stability with no aggregation and then, yet equally important, more reliable results. Overall, N/P = 10 uncoated micelleplexes showed the best performance, with SYBR Gold assay analysis indicating efficient siRNA encapsulation and satisfactory siRNA release (Figure 6c and Figure 6d). Indeed, “free siRNA” values approaching 0% indicate near-complete siRNA encapsulation within the micelleplexes. Conversely, in release assays, higher percentages of “free siRNA” correspond to greater siRNA dissociation from the complexes, with values above 50% considered indicative of adequate release capability.

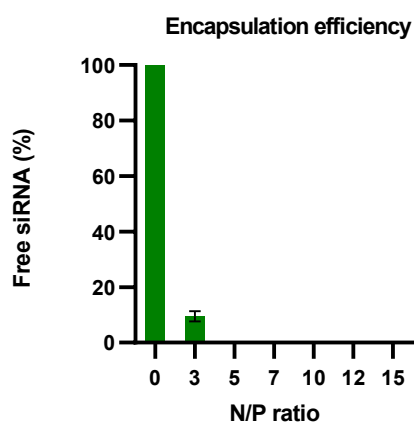
3.2 Physicochemical characterization of coated micelleplexes

3.2.1 Size and Zeta (ζ)-Potential analysis

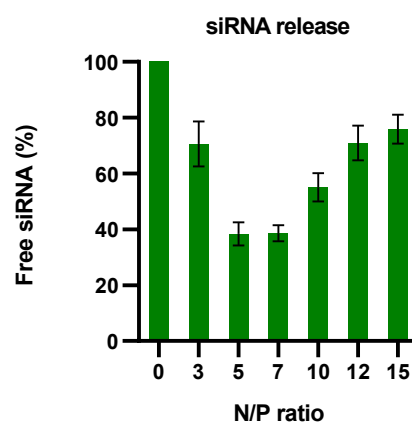
Particle size, PDI, and (ζ)-potential of all coated formulations were obtained by DLS and ELS, respectively. The optimal polymer coating should enhance stability and reduce the cytotoxicity of uncoated micelleplexes, while preserving their optimal physicochemical properties and transfection efficiency. For this reason, a comparative analysis in terms

(a) (ζ)-Potential values

(b) Hydrodynamic diameter and Polydispersity index (PDI)



(c) siRNA Encapsulation



(d) siRNA release

Figure 6: Physicochemical features of uncoated micelleplexes formulated at different N/P ratios. N/P = 0 was used as “free siRNA” control and does not lead to micelleplex formation due to the lack of electrostatic complexation between P(SpOABAE) and siRNA. (A) (ζ)-Potential, (B) Hydrodynamic diameter and Polydispersity index (PDI), (C) Encapsulation efficiency and (D) Release efficiency by SYBR Gold Assay.

of particle size, polydispersity index (PDI), and zeta potential was essential as a first assessment of the quality of each coated formulation. Ideally, the particle size should remain below 150 nm and the PDI below 0.3, as observed for the uncoated formulation, whose corresponding bars are reported in black in Figure 7. As illustrated in the same figure, most formulations, across all three investigated P(SpOABAE)-to-coating polymer molar ratios, maintained particle size and PDI values comparable to or lower than those of the uncoated formulation. The 2.5% low viscosity alginate, 2.5% medium viscosity alginate, and 0.5% PGA (15 kDa) coated formulations showed markedly increased and unacceptable hydrodynamic diameters, reaching 214 nm, 227.6 nm, and 797.3 nm, respectively. These suboptimal values, along with PDI values outside the acceptable range, are summarized in Table 2.

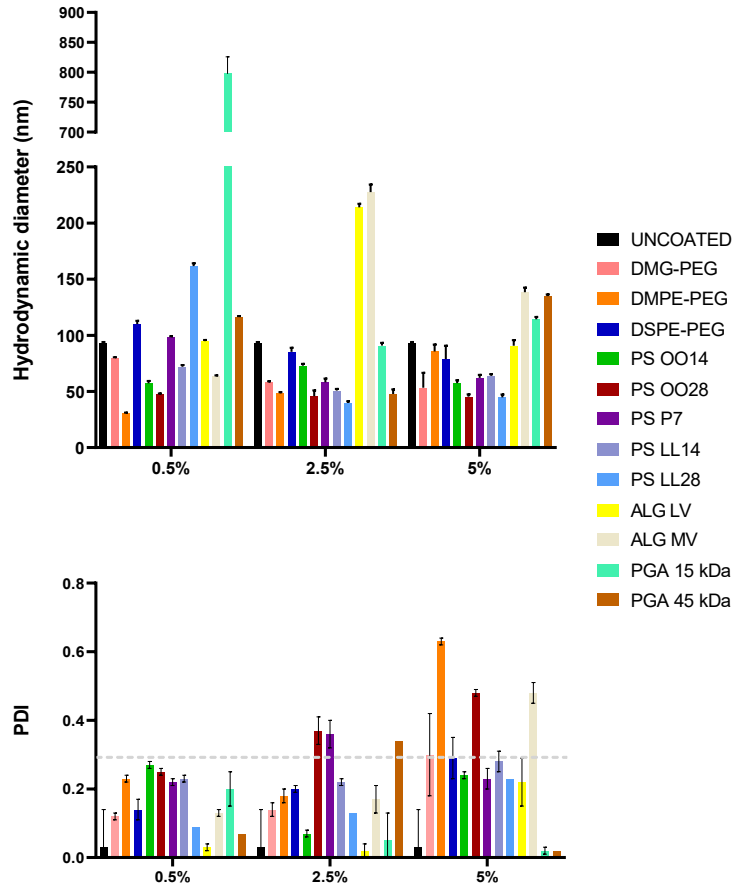


Figure 7: Hydrodynamic diameter (top panel) and polydispersity index (PDI, bottom panel) of all coated micelleplex formulations. Formulations are grouped on the x-axis according to the P(SpOABAE)-to-coating polymer molar ratio (0.5%, 2.5%, and 5%). The dashed grey line in the lower panel indicates the PDI threshold value ($PDI = 0.3$) to exclude formulations with higher dispersity.

As illustrated in Figure 8, all coated micelleplexes exhibited less positive zeta potential values compared to the uncoated formulation, suggesting a potential ability to shield the intrinsic positive charge of P(SpOABAE). Some formulations showed negative zeta potential values, which do not necessarily represent a limitation. The most negative zeta potential was recorded for 2.5% medium viscosity alginate (-16.57 mV), consistent with the intrinsically negative charge of the polymer.

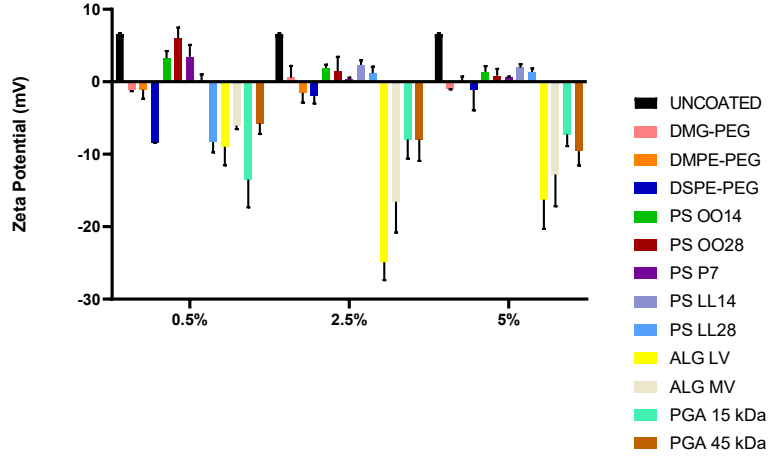


Figure 8: Zeta (ζ)-potential values of all coated micelleplex formulations. Formulations are grouped on the x-axis according to the P(SpOABAE)-to-coating polymer molar ratio (0.5%, 2.5%, and 5%).

3.2.2 siRNA encapsulation and release efficiency

Encapsulation efficiency of coated formulations was evaluated by SYBR Gold assay and expressed as the percentage of free siRNA, as shown on the y-axis in Figure 9. The striped bars corresponds to 100% free siRNA control. When micelleplexes achieve complete encapsulation, the percentage of free siRNA approaches zero. Accordingly, the black bars corresponding to the uncoated formulation are not visible, indicating negligible amounts of free siRNA. A similar behavior was observed for most of the coated formulations, as illustrated in the figure below (Figure 9). Unacceptable encapsulation efficiency values (on the order of 30% or higher) were observed for the 2.5% and 5% low-viscosity alginate and PGA (45 kDa) coated formulations, as reported in Table 2. This behavior may be attributed to the negatively charged nature of these coating polymers, which could preferentially interact with the cationic P(SpOABAE) chains, thereby competing with siRNA during micelleplex formation and reducing the overall encapsulation efficiency. Regarding release efficiency, micelleplexes exhibiting efficient siRNA release upon destabilization were expected to show fluorescence values comparable to the 100% free siRNA control or at least exceeding 60%. All the formulations fulfilled this criterion, except for DMPE-PEG 5% coated formulation (Figure 9, Table 2).

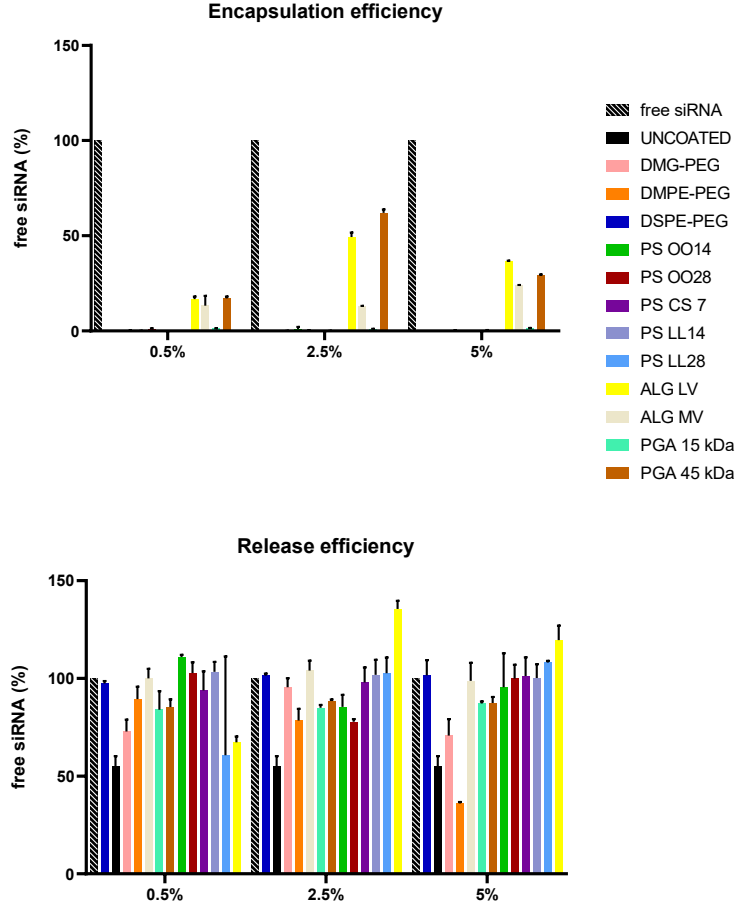


Figure 9: Encapsulation and release efficiency of all coated micelleplex formulations. Formulations are grouped on the x -axis according to the $P(\text{SpOABAE})$ -to-coating polymer molar ratio (0.5%, 2.5%, and 5%). The striped bars represent the 100% free siRNA control.

3.3 Time-dependent stability of micelleplexes in PBS

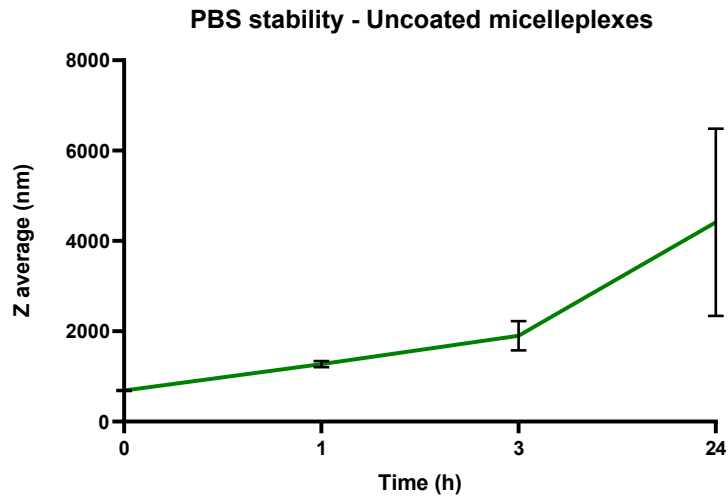
The ionic strength of the physiological environment can present a substantial risk to the stability of cationic delivery systems. Salts can interfere with the electrostatic interactions between the cationic carrier and the anionic nucleotide backbone, leading to aggregation [31]. Uncoated micelleplexes exhibited pronounced instability in PBS from the earliest time point (0 h), as shown in Figure 10. Their hydrodynamic size exceeded 600 nm immediately after dilution and continued to increase over time, reaching approximately 4414.33 nm after 24 h of incubation. Results concerning coated micelleplex formulations should show whether the shielding can provide protection against salt-induced aggregation through steric stabilization. This mechanisms has been consistently demonstrated for

Table 2: Summary of formulations not meeting the predefined physicochemical acceptance criteria.

Coating	pBAE: coating molar ratio	Non-compliant parameter	Experimental result
DMPE-PEG	5%	PDI, RE	0.63 ± 0.01 , 36.00 ± 0.75
PS P7	2.5%	PDI	0.36 ± 0.04
PS OO28	2.5%	PDI	0.37 ± 0.04
PS OO28	5%	PDI	0.48 ± 0.01
PGA 15 kDa	0.5%	Size	797.3 ± 28.67
PGA 45 kDa	2.5%	PDI, EE	0.34 ± 0.05 , 62.02 ± 1.75
PGA 45 kDa	5%	EE	29.15 ± 0.54
ALG LV	2.5%	Size, EE	214 ± 3.24 , 49.35 ± 2.33
ALG LV	5%	EE	36.45 ± 0.49
ALG MV	2.5%	Size	227.60 ± 6.80
ALG MV	5%	PDI	0.48 ± 0.03

Size: hydrodynamic diameter; PDI: polydispersity index; EE: encapsulation efficiency; RE: release efficiency. Only formulations not meeting the predefined acceptance criteria are reported.

PEGylated nanoparticles, due to PEG chains grafted on them generating a hydrated cloud with a large excluded volume that sterically prevents interparticle interactions [37].

**Figure 10:** Colloidal stability of uncoated micelleplexes in PBS over time

In line with this, all PEG-based coated micelleplex formulations maintained a hydrodynamic size below 150 nm throughout the analyzed time range, with no significant variation over time (Figure 11).

In contrast, most of the PGA- and alginate- coated formulations showed limited colloidal stability over time (Figure 12). In aqueous environments with low ionic strength, charged nanoparticles generally maintain colloidal stability due to electrostatic repulsion

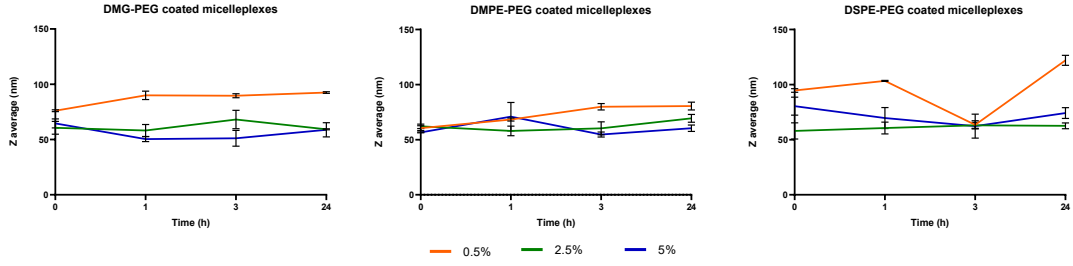


Figure 11: Colloidal stability of PEG-coated micelleplexes in PBS over time.

between them. However, under high ionic strength conditions, such as in PBS, interactions with counterions neutralize the particle surface charge, thereby rendering the NP surface amenable to aggregation [37]. Only 5% PGA (45 kDa), 2.5%, and 5% MV alginate-coated micelleplex formulations showed stability over time. This behavior may be attributed to the ability of the coating polymer, when present at higher molecular weight or concentration, to form a thicker layer around the micelleplexes, providing sufficient steric hindrance to prevent aggregation.

Polysarcosines (pSar) have already been widely proposed as a valid alternative to PEG for steric stabilization [22], due to their similar characteristics such as water solubility and no reported cytotoxicity and immunogenicity. Nevertheless, most polysarcosine-coated formulations exhibited a lack of colloidal stability in PBS over time, except for the 2.5% and 5% PS OO28-coated micelleplexes (Figure 12). This behavior may be attributed to the nature of pSar interaction with the polyplex surface. pSar interacts with P(SpOABAE) through weak, non-covalent interactions, including dipole-dipole interactions and hydrogen bonding. Under high ionic strength conditions, such as in PBS, these interactions may be weakened or disrupted due to charge screening and competition with ions in solution, potentially leading to partial desorption of the pSar coating. As a consequence, the steric barrier provided by pSar may be insufficient to prevent interparticle interactions, ultimately resulting in aggregation.

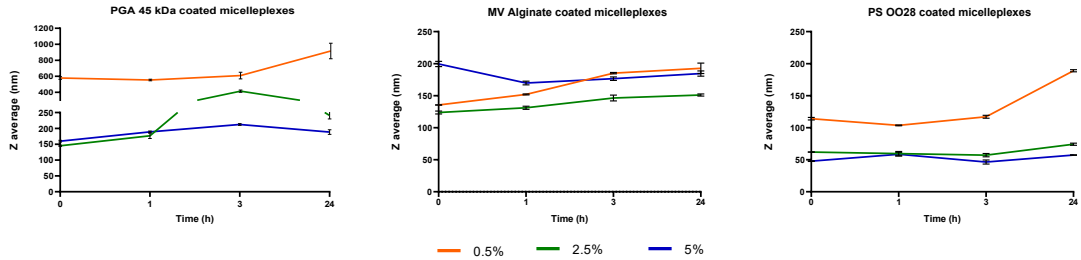


Figure 12: Colloidal stability of micelleplexes coated with PGA 45 kDa, MV Alginate, Polysarcosine OO28 in PBS over time (from left to right).

3.4 Assessment of micelleplex stability in the presence of Serum and SELF

Stability in Serum and Simulated Epithelial Lung Fluid was evaluated by analyzing the hydrodynamic size distribution curves of each formulation. These profiles were compared with the corresponding background controls, defined as the hydrodynamic size distribution curves obtained by DLS measurements of samples containing only serum or SELF, as described in the Materials and Methods Section 2.6. Examination of the hydrodynamic size distribution curves of the uncoated micelleplexes revealed a shift of the profile toward larger particle sizes beyond the main peak of the corresponding background curve under both analyzed conditions (incubation in serum and incubation in simulated epithelial lung fluid). This behavior may indicate the formation of larger aggregates upon incubation in serum and SELF, suggesting a lack of stability of the uncoated micelleplexes in these biological fluids. Conversely, hydrodynamic size distribution profiles overlapping with, or shifted toward smaller particle sizes relative to the main peak of the “background” curve may suggest preservation of colloidal stability under the analyzed condition. Although this assessment alone cannot be considered fully conclusive for definitively establishing colloidal stability, each coated formulation was evaluated in both biological fluids by applying the same analytical approach used for the uncoated micelleplexes. Based on these analyses, the formulations were classified according to their apparent stability behavior. Table 3 reports only the formulations that showed apparent stability in PBS and in at least one of the two biological fluids, also allowing the identification of potentially application-specific formulations. It is worth noting that none of the PS LL or PGA 15 kDa and LV Alginate-coated micelleplex formulations fulfilled the predefined stability requirements. Therefore, these formulations were excluded from subsequent biological testing.

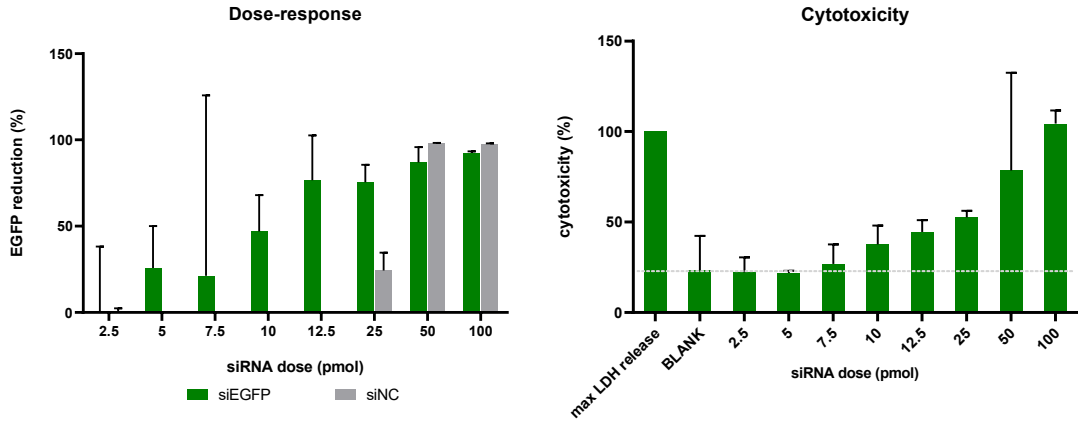
3.5 Dose-dependent gene silencing efficiency and cytotoxicity of micelleplexes

The gene silencing efficiency of micelleplexes was evaluated through eGFP knockdown experiments in Jurkat T cells. Following 48 h of incubation, cells were collected for flow cytometry analysis, while the corresponding supernatants were harvested to perform the LDH release assay for cytotoxicity evaluation. This approach allowed the simultaneous assessment of knockdown efficiency and formulation-associated cytotoxicity at each tested siRNA dose, initially for the uncoated formulation and subsequently for the selected coated formulations. In particular, the formulations carried forward for dose-dependent biological evaluations were selected from Table 3, excluding those that did not consistently satisfy the predefined physicochemical acceptance criteria (Table 2). The eligible formulations were DMG-PEG (0.5%, 2.5%, 5%)-, DMPE-PEG (0.5%, 2.5%)-, DSPE-PEG (0.5%, 2.5%, 5%)-, PS P7 5%-coated micelleplexes. To establish a preliminary dose-response curve, a range of siRNA doses was initially investigated (2.5, 5, 7.5, 10, 12.5, 25, 50, 100 pmol). Figure 13 shows the siRNA dose-response of the uncoated micelleplexes, alongside the corresponding formulation-associated cytotoxicity.

Table 3: Summary of the coated polyplex formulations exhibiting qualitative stability in PBS and in at least one of the two considered biological fluids (SERUM or SELF).

Coating	pBAE: coating molar ratio	Stability in PBS	Stability in SERUM or SELF
DMG-PEG	0.5%	✓	SERUM and SELF
DMG-PEG	2.5%	✓	SERUM and SELF
DMG-PEG	5%	✓	SELF
DMPE-PEG	0.5%	✓	SERUM and SELF
DMPE-PEG	2.5%	✓	SERUM and SELF
DMPE-PEG	2.5%	✓	SELF
DSPE-PEG	0.5%	✓	SELF
DSPE-PEG	2.5%	✓	SELF
DSPE-PEG	5%	✓	SERUM
PS CP7	5%	✓	SELF
PS OO28	2.5%	✓	SELF
PS OO28	5%	✓	SELF
PGA 45 kDa	5%	✓	SERUM
ALG MV	2.5%	✓	SELF
ALG MV	5%	✓	SELF

Uncoated micelleplexes

**Figure 13:** Dose-response profile and corresponding cytotoxicity of uncoated micelleplexes in Jurkat T cells following 48 h incubation. Left panel: siRNA dose-dependent eGFP reduction (%) assessed by flow cytometry. “siNC” bars represent control micelleplexes containing non-targeting siRNA. Right panel: formulation-associated cytotoxicity evaluated by LDH assay. The grey dashed line represents the baseline cytotoxicity level obtained from untreated cells. Data are presented as mean $\pm$ SD ($n=3$).

Both graphs clearly demonstrate a dose-dependent trend, as increasing siRNA doses resulted in a concomitant increase in both eGFP reduction and cytotoxicity. As expected, higher siRNA doses led to enhanced gene silencing efficiency, but were also associated

with increased formulation-related cytotoxicity. This behaviour is likely attributable to the higher amount of polymer required to complex increasing quantities of encapsulated siRNA.

At siRNA doses of 50 and 100 pmol, non-targeting siRNA (siNC) micelleplexes induced eGFP reduction levels comparable to those observed for siEGFP-loaded micelleplexes. Since no sequence-specific gene silencing is expected for the siNC control, this decrease in eGFP expression is likely attributable to the pronounced cytotoxic effects associated with these formulations. Indeed, excessive cellular stress may impair general metabolic activity and overall gene expression, resulting in an apparent reduction in eGFP signal independently of specific silencing mechanisms. This interpretation is further supported by the cytotoxicity data (Figure 13, right panel), where the cytotoxicity percentages at 50 and 100 pmol reached markedly elevated values, with the 100 pmol condition exceeding even the “max LDH release” control.

Moving to the coated formulations, the aim was to identify a polymer shielding strategy capable of improving the overall formulation performance while maintaining, or potentially enhancing, the transfection efficiency of the micelleplexes. Accordingly, coated formulations were expected to retain high eGFP reduction percentages while avoiding additional formulation-associated cytotoxicity.

Similar to what was observed for the uncoated micelleplexes at siRNA doses above approximately 50 pmol, the three DMG-PEG-coated formulations also exhibited non-specific eGFP reduction associated with increasing cytotoxicity. Notably, for the DMG-PEG 0.5% and 5% coated formulations, this effect was already evident at the lowest tested siRNA dose (2.5 pmol), where non-targeting siRNA micelleplexes induced substantial eGFP reduction. This behavior suggests that the coating may have enhanced the formulation-associated cytotoxicity, leading to non-specific gene silencing effects. Conversely, the DMG-PEG 2.5% coated micelleplexes showed a more favorable profile, with a dose-response trend closely resembling that of the uncoated formulation. This observation was also reflected in the corresponding cytotoxicity data, where cytotoxicity levels remained below, or close to, the baseline value of untreated cells (blank control) at all siRNA doses up to 25 pmol (Figure 14).

DMPE-PEG 0.5% and DMPE-PEG 2.5% coated micelleplexes did not yield better results, as both formulations exhibited increased cytotoxicity relative to untreated cells across almost all siRNA doses within the investigated range. Additionally, eGFP reduction was observed at all doses of non-targeting siRNA, further supporting the presence of a cytotoxicity-related effect, as previously discussed (Figure 15).

Among the DSPE-PEG coated formulations (Figure 16), the 2.5% and 5% coatings showed the most favorable balance between gene silencing efficiency and cytocompatibility. Up to a siRNA dose of 7.5 pmol, both formulations maintained eGFP knockdown levels comparable to those of the uncoated micelleplexes while exhibiting cytotoxicity values at or below those of the untreated control (“blank”). The DSPE-PEG 0.5% formulation displayed comparable performance only at the lowest tested dose (2.5 pmol).

Among the polysarcosine-coated formulations, PS P7 5% was the only candidate selected for further biological testing, as it was the only formulation meeting the predefined physicochemical and stability acceptance criteria. The corresponding results (Figure 17)

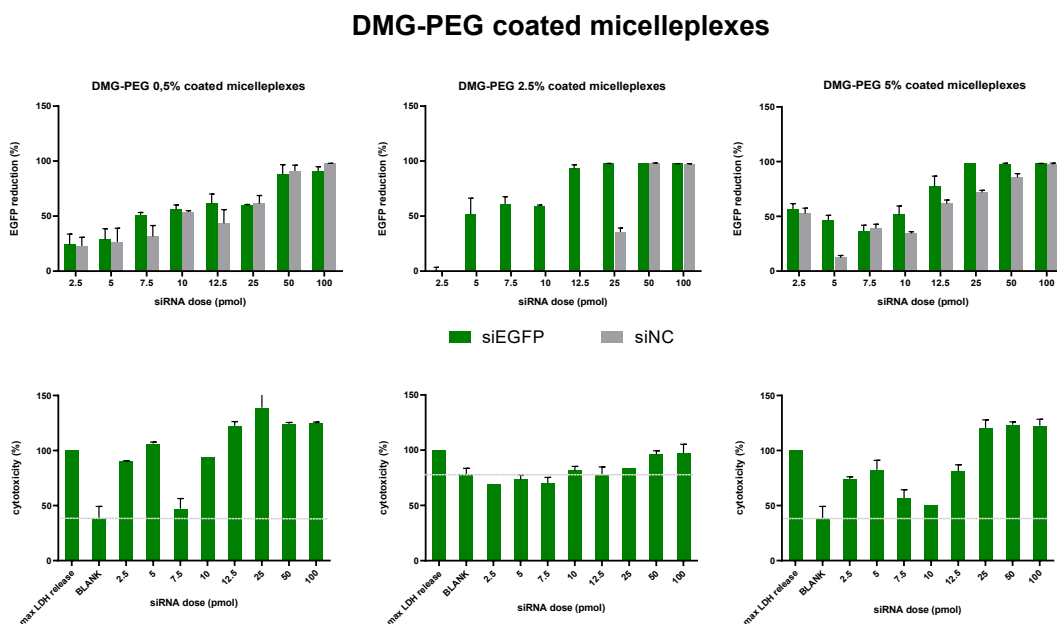


Figure 14: Dose-response profile and corresponding cytotoxicity of DMG-PEG coated micelleplexes in Jurkat T cells following 48 h incubation. In the upper panels, “siNC” bars represent control micelleplexes containing non-targeting siRNA. In the lower panels, the grey dashed line represents the baseline cytotoxicity level obtained from untreated cells. Data are presented as mean $\pm$ SD ($n=3$).

revealed two particularly promising formulations, corresponding to siRNA doses 2.5 and 5 pmol. Indeed, despite the eGFP reduction (%) remaining below 50% in both cases, the performance remained comparable, if not slightly improved, relative to the uncoated formulation at the same siRNA doses. No relevant gene silencing effect was observed for non-targeting siRNA micelleplexes at these doses, further supporting the low associated cytotoxicity observed in the corresponding cytotoxicity data (Figure 17), with values remaining below the baseline level of untreated cells (blank control).

It is important to note that cytotoxicity values were obtained under T-cell activation conditions induced by PMA and ionomycin. As these stimulatory conditions are associated with increased cellular stress, the elevated baseline cytotoxicity relative to the blank may also be attributable to the activation conditions and should be considered when interpreting formulation-associated effects.

3.6 Hemolytic activity of micelleplexes

Based on the cytotoxicity results discussed in Section 3.5, the 100 pmol siRNA dose was excluded from the subsequent hemolysis assay. Hemolytic activity of micelleplexes was evaluated through an erythrocyte lysis assay, beginning with the uncoated formulation as a reference for subsequent comparisons. The uncoated micelleplexes exhibited negligible

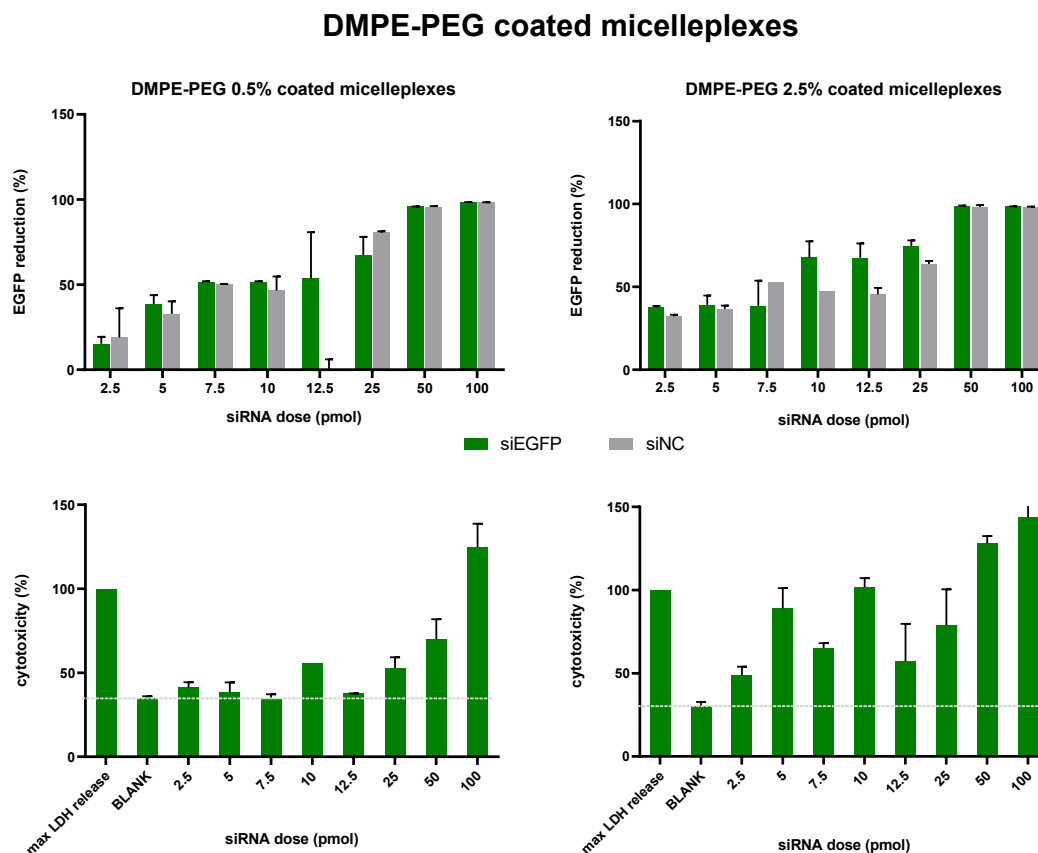


Figure 15: Dose-response profile and corresponding cytotoxicity of selected DMPE-PEG coated micelleplexes in Jurkat T cells following 48 h incubation. In the upper panels, “siNC” bars represent control micelleplexes containing non-targeting siRNA. In the lower panels, the grey dashed line represents the baseline cytotoxicity level obtained from untreated cells. Data are presented as mean $\pm$ SD ($n = 3$).

hemolytic activity across the entire investigated siRNA dose range, with the maximum hemolysis percentage reaching only approximately 13% at the maximum siRNA dose, as shown in Figure 18. A comparable behavior was observed for all the biologically tested coated formulations, with hemolysis values remaining below 30% across the entire investigated siRNA dose range. These results indicate favorable hemocompatibility, suggesting that the investigated formulations induce only limited erythrocyte membrane disruption and may therefore represent suitable candidates for systemic administration.

Overall, the coating strategy did not appear to compromise micelleplex hemocompatibility, as limited hemolysis percentages were generally maintained across all coated formulations and investigated siRNA doses. Nevertheless, at the highest siRNA dose (50 pmol), increased hemolytic activity was detected for the DSPE-PEG 2.5% and DSPE-PEG 5% coated formulations, reaching potentially unacceptable values above 50% (Figure 19).

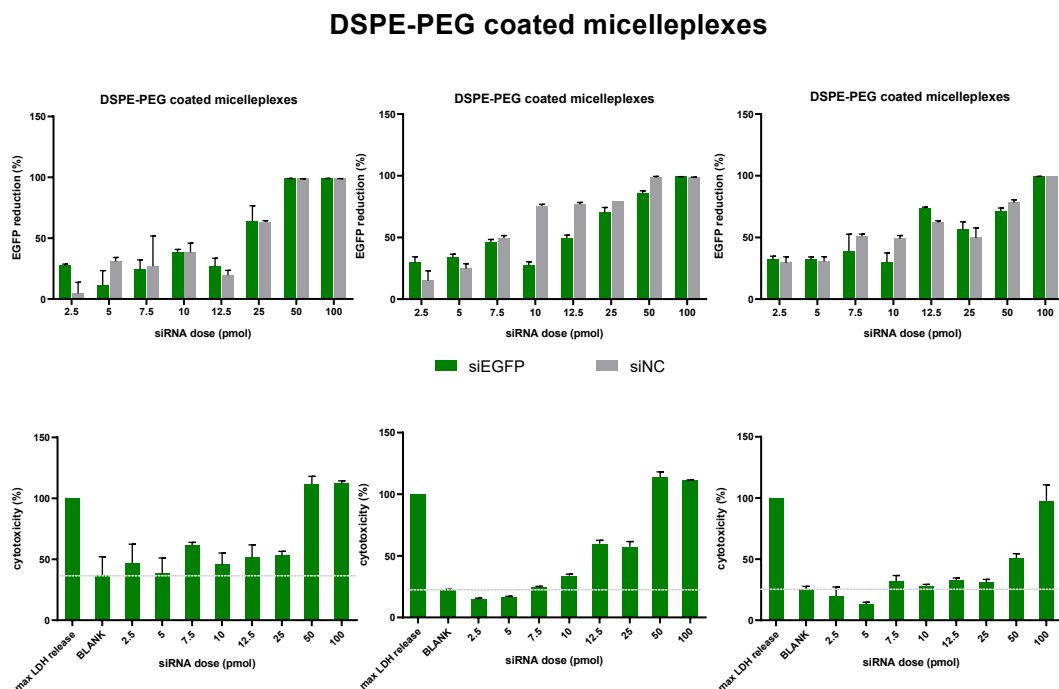


Figure 16: Dose-response profile and corresponding cytotoxicity of DSPE-PEG coated micelleplexes in Jurkat T cells following 48 h incubation. In the upper panels, “siNC” bars represent control micelleplexes containing non-targeting siRNA. In the lower panels, the grey dashed line represents the baseline cytotoxicity level obtained from untreated cells. Data are presented as mean $\pm$ SD ($n = 3$).

Considering that doubling the siRNA dose from 25 to 50 pmol also resulted in a higher amount of P(SpOABAE) in the final polyplex formulation, these findings suggest that the combination of higher polymer content and elevated P(SpOABAE)-to-DSPE-PEG molar ratios (respectively 1:0.025 and 1:0.05) may promote less favorable interactions with erythrocyte membranes.

3.7 Evaluation of transferrin-conjugated micelleplexes

Since only DSPE-PEG-Azide was available, this type of coated micelleplex formulation was initially subjected to transferrin conjugation. Indeed, the terminal azide functional groups ($-N_3$) enabled the covalent attachment of transferrin through azide-alkyne click chemistry, following the conjugation procedure described in Section 2.11. Since all investigated DSPE-PEG formulations, corresponding to different P(SpOABAE)-to-coating polymer molar ratios (0.5%, 2.5%, and 5%), had previously fulfilled the physicochemical and stability criteria established during formulation screening, all were considered for this investigation. As a first step, it was evaluated whether transferrin conjugation could affect the physicochemical characteristics of the micelleplexes. Figure 20 shows the results in

PS Pal 7 5% coated micelleplexes

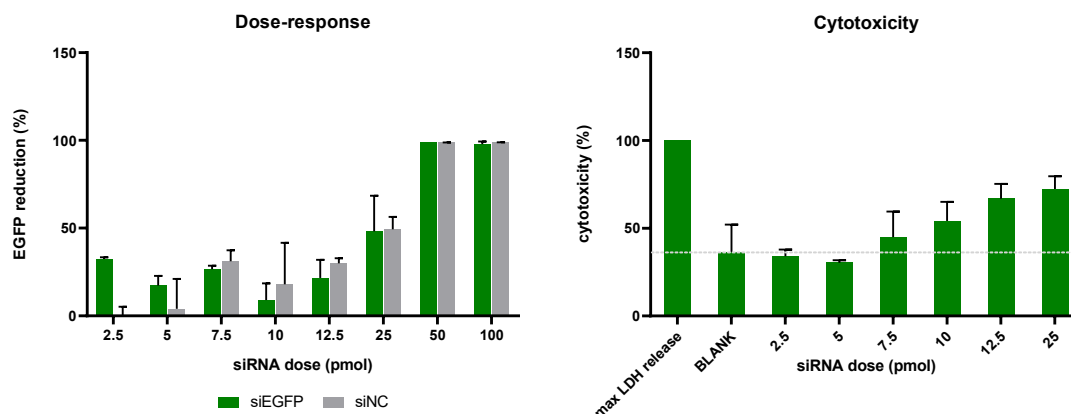


Figure 17: Dose-response profile and corresponding cytotoxicity of PS C7 5% coated micelleplexes in Jurkat T cells following 48 h incubation. Left panel: siRNA dose-dependent eGFP reduction (%) assessed by flow cytometry. “siNC” bars represent control micelleplexes containing non-targeting siRNA. Right panel: formulation-associated cytotoxicity evaluated by LDH assay. The grey dashed line represents the baseline cytotoxicity level obtained from untreated cells. Data are presented as mean $\pm$ SD ($n = 3$).

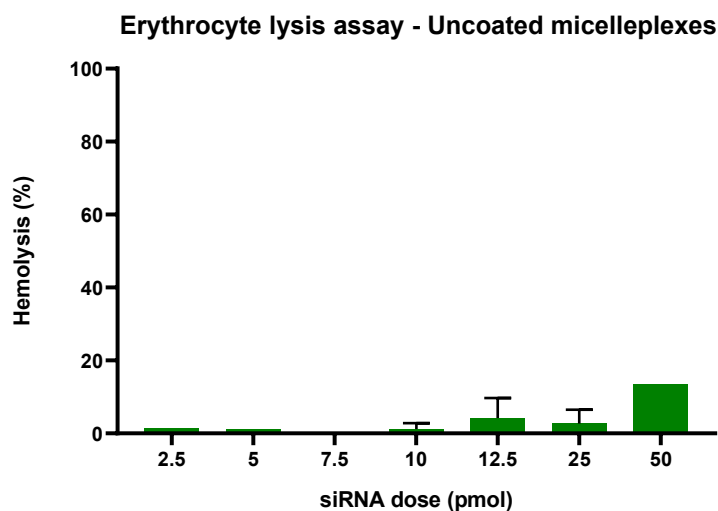


Figure 18: Hemolytic activity of uncoated micelleplexes evaluated by erythrocyte lysis assay at increasing siRNA doses. Hemolysis percentages indicate the degree of erythrocyte membrane disruption induced by the formulation, calculated according to Equation (2.5). Data are presented as mean $\pm$ SD ($n = 3$).

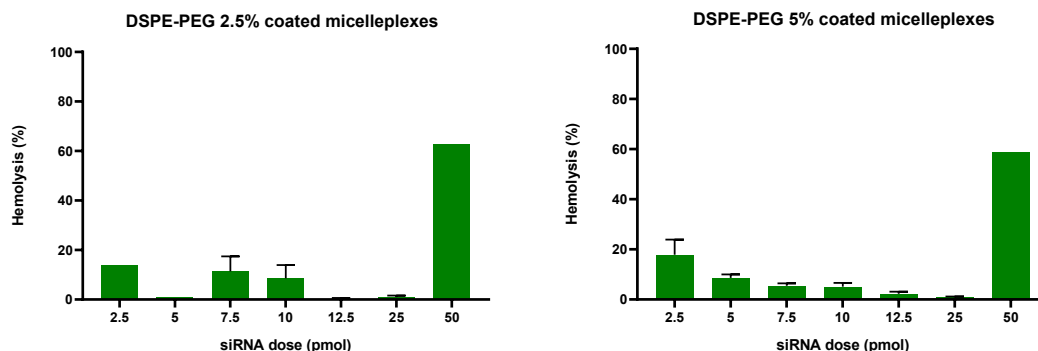


Figure 19: Hemolytic activity of DSPE-PEG 2.5% and DSPE-PEG 5% coated micelleplexes, whose hemolysis percentages deviated from the overall low-hemolysis trend observed for uncoated and the other coated formulations investigated. Data are presented as mean $\pm$ SD ($n = 3$).

terms of size, surface charge, EE and RE efficiency, comparing the transferrin-conjugated and non-conjugated formulations in order to identify any significant variations.

As shown in the lower panels of Figure 20, transferrin conjugation did not negatively affect either encapsulation efficiency (EE) or release efficiency (RE), with all investigated formulations maintaining values within the previously established acceptance criteria. Regarding size and surface charge (top panels, Figure 20), hydrodynamic diameter values remained overall comparable following conjugation, although reduced particle sizes were observed for the 2.5% and 5% formulations. Considering that the molar amount of transferrin used for conjugation corresponded to the DSPE-PEG quantity associated with the 0.5 mol% formulation, higher coating polymer-to-pBAE molar ratios likely provided a larger number of available azide functional groups for conjugation. This may have promoted a higher transferrin surface functionalization density, potentially favoring a more compact organization of the outer layer and consequently lower hydrodynamic diameter values. In terms of surface charge, the 0.5% transferrin-conjugated formulation exhibited a markedly more negative zeta potential compared to the corresponding non-conjugated formulation. This behavior may be related to the intrinsic negative charge of transferrin, whose contribution to the overall surface charge could become more pronounced at lower DSPE-PEG coating densities. In contrast, comparable zeta potential values were observed between transferrin-conjugated and non-conjugated formulations at the 2.5% and 5% P(SpOABAE)-to-coating polymer molar ratios. Furthermore, PDI values remained below 0.3 for all investigated transferrin-conjugated micelleplex formulations, with no remarkable variations compared to the corresponding non-conjugated ones.

With regard to colloidal stability in PBS over time, Figure 21 shows that transferrin-conjugated and non-conjugated formulations exhibited a highly comparable stability trend, suggesting that transferrin conjugation did not affect the ability of the DSPE-PEG coating, and likely PEG-based coatings in general, to prevent salt-induced aggregation through steric stabilization.

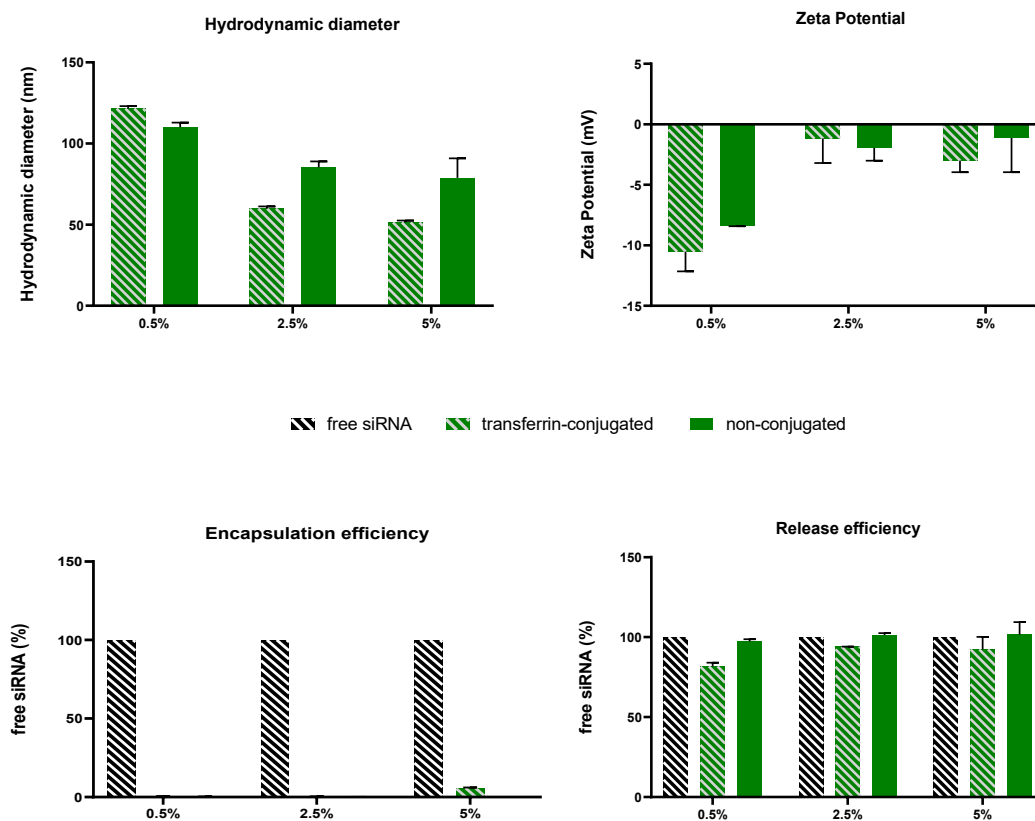


Figure 20: Comparison of the physicochemical characteristics of transferrin-conjugated and non-conjugated micelleplexes in order to evaluate the impact of transferrin conjugation on hydrodynamic diameter, zeta potential, encapsulation efficiency (EE) and release efficiency (RE). Data are presented as mean $\pm$ SD ($n=3$).

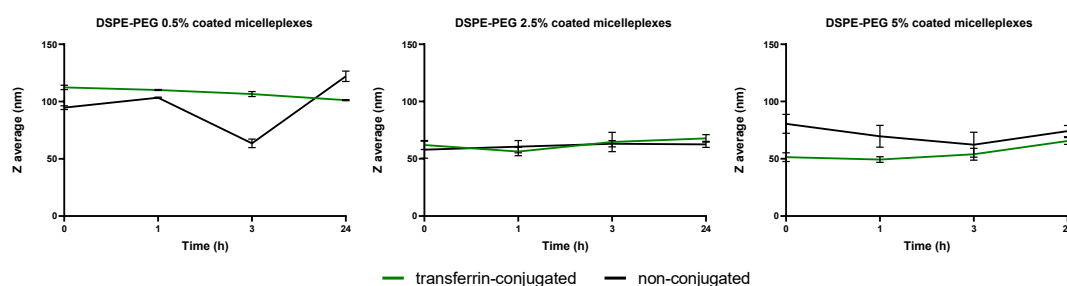


Figure 21: Comparison of the time-dependent size profiles upon PBS addition of transferrin-conjugated and non-conjugated formulations. From left to right: DSPE-PEG 0.5%, DSPE-PEG 2.5%, and DSPE-PEG 5% coated micelleplexes. Data are presented as mean $\pm$ SD ($n=3$).

Since the serum and SELF stability investigations were evaluated qualitatively, only limited conclusions could be drawn. However, all transferrin-conjugated DSPE-PEG

coated micelleplex formulations exhibited stability in PBS and in at least one of the two investigated biological fluids, except for the 2.5% formulation, which appeared to be stable only in PBS.

The knockdown efficiency of the non-conjugated and corresponding conjugated micelleplexes was subsequently evaluated. Results are reported in Figure 22.

According to what has already been discussed regarding the knockdown efficiency associated with the non-targeting siRNA, the 5% transferrin-conjugated formulation appeared to be excessively cytotoxic at each siRNA dose. For the other two P(SpOABAE)-to-coating polymer molar ratios, an apparently unexpected trend could be observed: transferrin conjugation seemed to almost completely abolish gene silencing efficiency across nearly the entire siRNA dose range, with the exception of the 50 and 100 pmol doses. However, these siRNA doses were consistently associated with unacceptable cytotoxicity across all formulations investigated throughout the study. The only transferrin-conjugated formulation that appears promising is the 0.5% formulation at the 5 pmol siRNA dose, which shows enhanced knockdown capability while apparently maintaining lower cytotoxicity compared with its non-conjugated counterpart. Nevertheless, although this formulation at this specific dose preserves the favorable performance observed for the uncoated formulation, the result appears to behave as an outlier, since siRNA doses within the same range do not follow the trend that would naturally be expected.

No substantial differences were detected in the erythrocyte lysis assay following transferrin conjugation. Regardless of the P(SpOABAE)-to-DSPE-PEG molar ratio, all transferrin-conjugated formulations maintained hemolysis (%) values similar to those previously discussed for the non-conjugated counterparts (Section 3.6). These findings indicate that transferrin conjugation does not affect the hemocompatibility of the micelleplexes.

3.8 Cell viability of transferrin-conjugated micelleplexes

Based on the previous screening results and starting from the formulation characterized by a 1:1 molar ratio of transferrin to the coating polymer, DSPE-PEG 0.5% coated micelleplexes were first evaluated using the CellTiter-Glo[®] Luminescent Cell Viability Assay. The analysis aimed to assess potential differences in the number of viable cells following treatment with uncoated micelleplexes, coated micelleplexes, and transferrin-conjugated coated micelleplexes. As shown in Figure 23, similar viability percentages were observed for both uncoated and transferrin-conjugated coated micelleplexes at each siRNA dose. No substantial differences were detected for non-transferrin-conjugated coated micelleplexes either, suggesting that coating and transferrin conjugation do not increase cell death. Moreover, although cell viability would be expected to decrease with increasing siRNA doses due to the higher polymer amounts required for complexation, the percentage of viable cells remained above 50% under all tested conditions, even at the highest siRNA concentrations. These promising results in terms of cell viability allow for a partial reinterpretation of the cytotoxicity data, taking into account the nature of the assay. Although LDH release is commonly used as a measure of cytotoxicity, it primarily serves as a marker of membrane integrity loss [38]. In the case of polycationic nanoparticles, the same membrane-interacting properties that facilitate endosomal escape can induce

transient pores or destabilization of the plasma membrane [39]. Even though the micelleplex surface is near-neutral, the cationic P(SpOABAE) core can become protonated in response to acidic pH in inflamed microenvironments, becoming activated at the target site and thereby enabling the same membrane-interacting effect [40]. Small amounts of LDH may leak through these temporary disruptions; however, the cells remain metabolically active with preserved ATP levels. Moreover, cells can respond to membrane stress by increasing mitochondrial activity [38]. This repair response can paradoxically elevate ATP levels in surviving cells, thereby reconciling the two readouts.

The high eGFP knockdown percentages associated with non-targeted siRNA, as reported in Section 3.5, may similarly reflect transient membrane stress. At the same time, the preservation of ATP levels indicates that mitochondria are still functional and that the cells maintain sufficient energy to repair damage and support metabolic activity, as previously mentioned. However, to accurately determine whether the overall elevated LDH levels reflect transient membrane impairment or actual cell death, it would be necessary to perform both assays in parallel under identical culture conditions and at multiple consistent time points.

Elevated ATP levels 24 hours after incubation with micelleplexes may indicate that the cells have not yet undergone lethal damage and are actively repairing. However, additional ATP measurements at 48 hours, together with LDH measurements collected at the same time points, would be necessary to accurately interpret the observed cytotoxicity profile. High LDH levels after 48 hours, if accompanied by low ATP levels, would suggest that the cells are undergoing apoptosis or necrosis. In such a scenario, the combined interpretation of the two measurements would suggest that the cytotoxicity associated with the micelleplexes is primarily due to irreversible membrane disruption.

3.9 Effect of transferrin conjugation on micelleplex uptake in CD4⁺ T cells

For the same reasons reported in Section 3.8, the formulation evaluated in this study consisted of DSPE-PEG 0,5% coated micelleplexes. Specifically, the unconjugated formulation and its transferrin-conjugated counterpart were investigated to determine whether transferrin functionalization could enhance cellular uptake. Two experimental conditions were analyzed: non-activated CD4⁺ T cells (right panel, Figure 24) and activated CD4⁺ T cells (left panel, Figure 24). Upon activation, T cells upregulate the transferrin receptor TfR1/CD71, potentially enhancing transferrin-mediated uptake of micelleplexes.

Cellular uptake of the “free siRNA” sample remained low under both activated and non-activated conditions, indicating that naked siRNA is poorly internalized in the absence of a delivery carrier. Under non-activated conditions, the uptake levels observed for both transferrin-conjugated and non-conjugated micelleplexes were comparable to those measured for “free siRNA”. This suggests that, independently of transferrin functionalization, these formulations are not efficiently internalized by resting T cells. Since the micelleplexes were designed to preferentially target activated T cells, which display increased metabolic activity and enhanced TfR1/CD71 expression, these results support the selective uptake of the formulations in activated T cells.

Under activated conditions, transferrin-mediated uptake was markedly enhanced compared to the internalization of non-conjugated micelleplexes. Specifically, transferrin-conjugated micelleplexes showed cellular uptake values of approximately 51%, whereas non-conjugated formulation remained close to 12%, indicating substantially increased internalization in activated T cells. Moreover, the cellular uptake achieved by transferrin-conjugated micelleplexes approached that of the Lipofectamine 2000 positive control, further supporting the potential of this formulation as an efficient targeted delivery system.

The marked decrease in eGFP knockdown observed for transferrin-conjugated micelleplexes (Section 3.7), despite their substantially increased cellular uptake, suggests that transferrin conjugation enhanced cell internalization but impaired the intracellular bioavailability of siRNA. Since the SYBR Gold assay confirmed that siRNA release was not affected by transferrin-conjugation, the reduced eGFP knockdown is more likely related to altered intracellular trafficking rather than insufficient siRNA dissociation from the carrier. In particular, TfR-mediated uptake may direct the micelleplexes toward recycling endosomal pathways [25], while transferrin conjugation may impair endosomal escape by reducing micelleplex-endosomal membrane interactions [41]. Both mechanisms could potentially limit effective cytosolic siRNA delivery despite the enhanced cellular uptake.

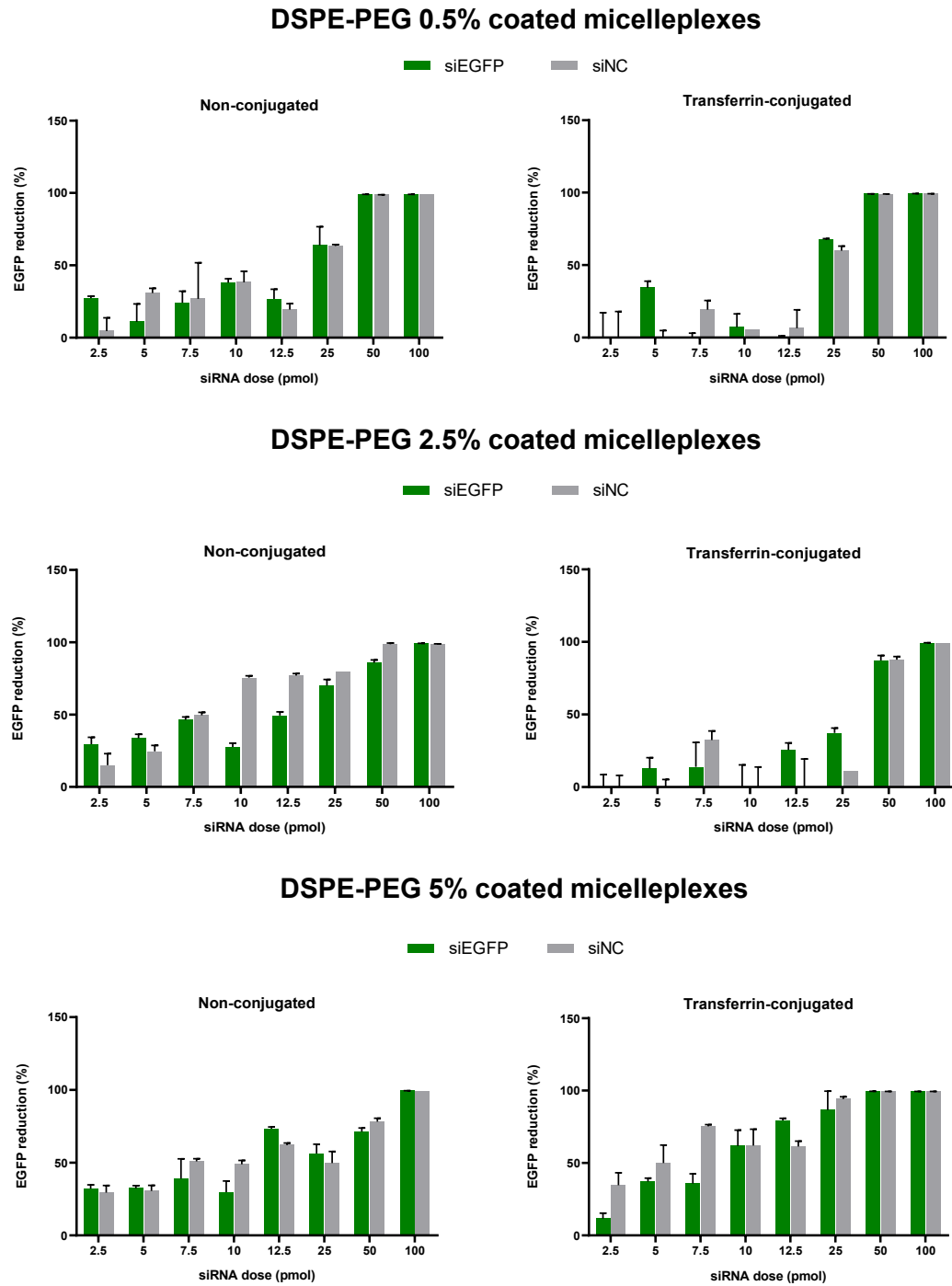


Figure 22: siRNA dose-dependent eGFP reduction (%) assessed by flow cytometry. Comparison of the dose-response profiles of DSPE-PEG-coated micelleplexes and their transferrin-conjugated counterparts. "siNC" bars represent control micelleplexes containing non-targeting siRNA. Data are presented as mean $\pm$ SD ($n = 3$).

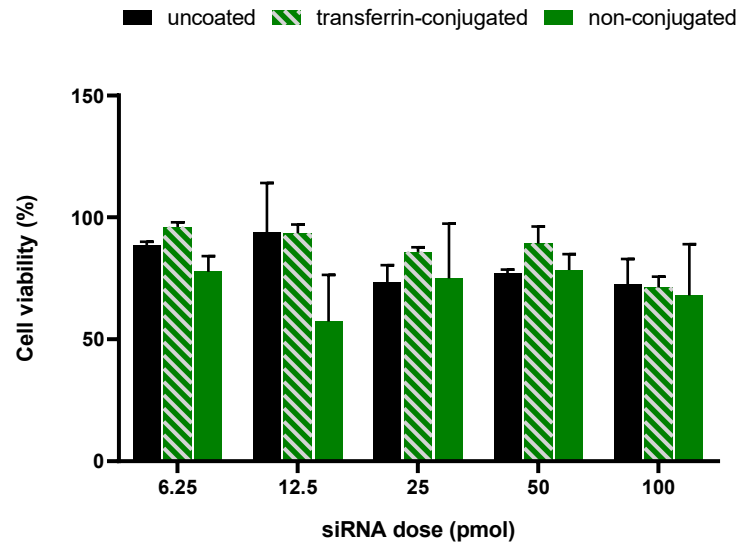


Figure 23: Cell viability of cells treated with uncoated micelleplexes, coated micelleplexes, and transferrin-conjugated coated micelleplexes at increasing siRNA doses (6.25-100 pmol) after 24 h incubation. Data are presented as mean $\pm$ SD ($n=3$).

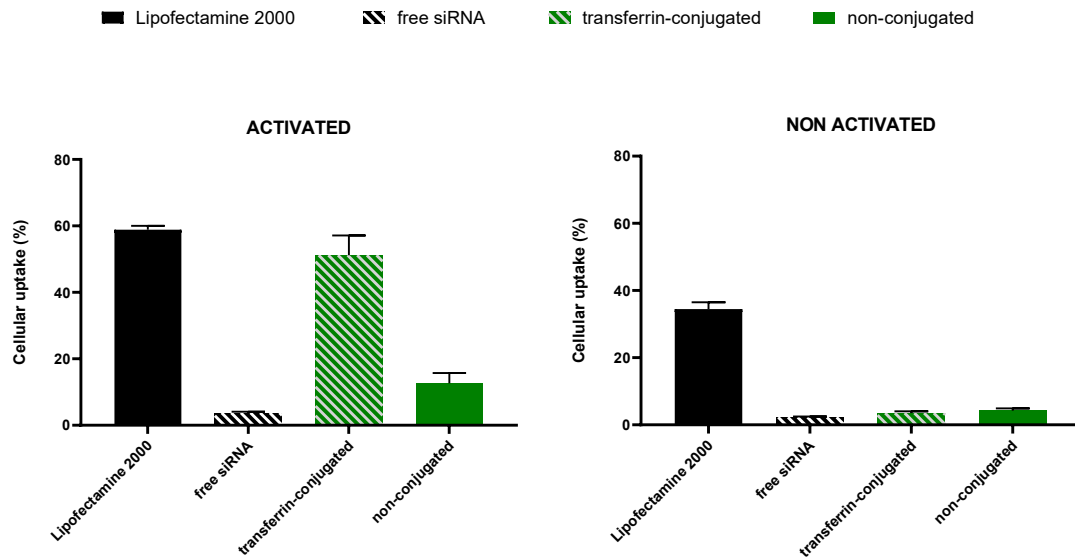


Figure 24: Percentage of AF488-positive $CD4^+$ T cells in activated and non-activated conditions after treatment with DSPE-PEG 0.5%-coated micelleplexes. Cellular uptake of transferrin-conjugated and non-conjugated formulations was compared with Lipofectamine 2000 (positive control) and free siRNA as an indicator of passive uptake. Data are presented as mean $\pm$ SD ($n=3$).

Chapter 4

Conclusion and Outlook

P(SpOABAE)-based micelleplex formulations were prepared, optimized, and evaluated for targeted siRNA delivery to activated immune cells. Different polymeric coatings were investigated to improve the stability and reduce the cytotoxicity of uncoated P(SpOABAE) micelleplexes. All polymeric coatings tended to reverse the intrinsic positive surface charge of P(SpOABAE) micelleplexes, thereby potentially mitigating the cytotoxicity associated with cationic formulations. Most coated formulations also preserved the other favorable physicochemical properties of uncoated micelleplexes, maintaining high encapsulation efficiency and efficient siRNA release profiles while retaining particle sizes within the optimal range. Stability studies in physiologically relevant media indicates aggregation of uncoated micelleplexes in PBS, FBS, and SELF. In contrast, all PEGylated lipid coatings, as well as the 5% PSar Cap-Ser(Pal)-Sar7-NH₂ coated formulation, appeared to improve apparent stability in PBS. Their behavior in FBS and SELF also allowed these formulations to be identified as promising candidates for IV injection or inhalation administration, although further studies are required to confirm this hypothesis. The different stability behavior observed for all other polysarcosines-, PGA-, and alginate-based coatings may be related to weaker interactions with the micelleplex surface, resulting in shielding layers that were easily destabilized under biological conditions. The formulations exhibiting enhanced apparent colloidal stability were subsequently advanced for further biological testing to identify polymeric coatings capable of maintaining or enhancing the transfection efficiency of uncoated micelleplexes while preserving low cytotoxicity. Nevertheless, all tested coated formulations exhibited markedly higher cytotoxicity compared to the uncoated formulation. The DMG-PEG 2.5% coated micelleplex formulation emerged as the only biologically promising system, demonstrating effective knockdown together with minimal toxicity at siRNA doses of 5, 7.5, 10, 12.5, and 25 pmol. Low hemolysis levels were observed for all selected formulations, suggesting good hemocompatibility of the micelleplexes and further supporting their potential for systemic administration. Regarding transferrin conjugation for targeted siRNA delivery, transferrin functionalization of the micelleplexes did not negatively affect any of their physicochemical characteristics, including stability in PBS. Hemocompatibility was likewise preserved. However, none of the transferrin-conjugated formulations demonstrated satisfactory transfection efficiency. This result was not fully consistent with the cellular

uptake data, which showed that transferrin-conjugated micelleplexes achieved approximately 40% higher uptake in activated T cells compared with the non-conjugated formulation. These findings suggest that transferrin functionalization substantially enhanced cellular uptake, potentially through receptor-mediated mechanisms. However, this did not translate into efficient cytosolic siRNA delivery and subsequent target mRNA silencing. Future studies should also include the other two formulations, namely the DSPE-PEG 2.5% and 5% coated micelleplexes, despite their less favorable performance compared with the 0.5% formulation in the present work. This would help clarify whether the density of the polymeric coating influences transferrin functionalization and, consequently, cellular uptake. Furthermore, the amount of transferrin conjugated to the systems could also be varied by testing molar amounts equal to or higher than those of the coating, to better understand how transferrin density affects the efficiency of conjugation and the overall delivery performance. The cell viability assay should also be performed for the 2.5% and 5% DSPE-PEG coated formulations, while the 0.5% formulation should be re-evaluated, with all formulations being tested under identical culture conditions and at multiple time points consistent with those adopted for the LDH release cytotoxicity assay. In this way, the results obtained from the two assays could be interpreted as complementary and provide a more comprehensive evaluation of formulation-associated cytotoxicity. Based on the findings of the present study, however, any polymer-coated formulation characterized by a 5 mol% pBAE-to-coating polymer molar ratio is still expected to exhibit excessive cytotoxicity, likely due to the high polymer content. Investigating different polymer coatings and coating densities opened new possibilities for optimizing already efficient delivery systems by improving their performance in biological environments. Overall, this study demonstrates the broad potential of polymer coatings not only to improve micelleplex stability, but also to enable functionalization with targeting agents. At the same time, it also emphasizes the critical balance between colloidal stability, targeting efficiency, biocompatibility, and efficient gene silencing. The results obtained in this work provide a solid foundation for future studies focused on the further optimization of targeted siRNA delivery systems.

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