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Engineered siRNA-loaded nanocarriers for CCR5 targeting and silencing

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“Não tenhamos pressa,

mas não percamos tempo.”

José Saramago

Abstract

Human immunodeficiency virus (HIV) infection, the cause of acquired immune deficiency syndrome (AIDS), continues to be a worldwide health issue, since it is still a significant cause for mortality and morbidity all around the globe. While an effective and safe treatment is still searched for, prevention of the infection has gained a vital role in the combat against HIV/AIDS. Particularly, topical microbicides, for both vaginal and rectal use, have been the focus of intensive research. An approach that has attracted significant attention in this context is nanotechnology. Nanosystems, which allow the targeted delivery of compounds, might present themselves as extremely promising, as they would allow specific delivery to targets involved in early phases of HIV infection. This work aims at developing polymeric-based nanoparticles (NPs) capable of incorporating specific siRNAs targeting the C-C chemokine receptor type 5 (CCR5). Such nanosystems may pose as an innovative antiretroviral approach targeting HIV-susceptible cells that are typically involved in early viral transmission at the cervicovaginal and colorectal mucosae.

To establish the ability of a commercial siRNA to silence CCR5 expression, several transfections using different transfection agents were performed. When this siRNA was found not to properly reduce the levels of this receptor, a different commercial siRNA, with a distinct nucleotide sequence, was tested, and was able to knockdown in significant fashion CCR5 expression. Receptor expression levels were assessed by Fluorescence-Activated Cell Sorting (FACS). This siRNA, either by itself or pre-complexed with polyamines, was then incorporated in NPs, which were developed by using poly(lactic-co-glycolic acid) (PLGA)-polyethylene glycol (PEG) copolymers and poloxamer 407 as a surfactant. NPs were produced by double emulsion solvent evaporation technique. NP size (mean hydrodynamic diameter) and surface charge (zeta potential) were determined using Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS), respectively. All NP formulations had average hydrodynamic diameter values between 120 nm and 180 nm and were relatively monodisperse (polydispersity index values below or close to 0.1). Zeta potential values ranging from -8 to –3 mV indicated that NPs presented nearly neutral surface charge.

Studies undertaken to assess the amount of siRNA present in each formulation, and the respective association efficiencies, through fluorescence revealed to be unsuccessful, considering the results obtained. However, a tendency of higher association for siRNA complexed with a polyamine could be observed.

Some NPs formulations demonstrated similar capability of delivering siRNA and silencing CCR5 when compared to commercial transfection agents. In fact, NPs associated with siRNA complexed with spermidine, one of the tested polyamines, lead to a significant decrease in the receptor levels compared to both untreated and treated with blank NPs cells.

In conclusion, siRNA-containing PLGA-PEG NPs were successfully produced and characterized for relevant physicochemical and biological properties. In particular, obtained data suggested that functionalized NPs present suitable general features for potential use in microbicide development. However, successful association of siRNA within the formulation was not demonstrated and additional studies are required. In spite of that, some of the developed formulations were capable of inducing a similar effect regarding CCR5 expression when compared to commercially available transfection agents.

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Abbreviations

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AAV	Adeno-associated virus
AIDS	Acquired immunodeficiency syndrome
ANOVA	One-way analysis of variance
APC	Allophycocyanin
bNAbs	Broadly neutralizing antibodies
Cas9	CRISPR associated protein 9
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CD195	Cluster of differentiation 195
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CXCR4	C-X-C chemokine receptor type 4
DAPTA	D-ala-peptide T-amide
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA

EDTA	Ethylenediamine tetraacetic acid
ELS	Electrophoretic Light Scattering
EMA	European Medicines Agency
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
FSC-A	Forward scatter area
FSC-H	Forward scatter height
gp120	Glycoprotein 120
HIV	Human immunodeficiency virus
miRNA	Micro RNA
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVC	Maraviroc
Mw	Molecular weight
MWCO	Molecular weight cut-off
NP	Nanoparticle
N/P	Nitrogen/Phosphate
PBS	Phosphate buffered saline
PdI	Polydispersity index
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLGA	Poly(lactic-co-glycolic acid)
PrEP	Pre-Exposure Prophylaxis
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic acid
RNAi	RNA interference
SD	Standard deviation
shRNA	Short hairpin RNA

siRNA	Small interference RNA
SSC-A	Side scatter area
ssDNA	Single stranded DNA
UNAIDS	Joint United Nations Program on HIV/AIDS

CHAPTER 1

Objective and Document Structure

1.1 Objective and rationale

The objective of this work was to develop polymeric-based nanoparticles (NPs) capable of carrying and delivering small interference RNA (siRNA) targeting the C-C chemokine receptor type 5 (CCR5), a receptor involved in HIV entry into susceptible cells. Promising nanosystems emerging from this work are intended to be considered in the future as potential antiretroviral agents in the context of microbicide development.

For the purpose of this work, a poly(lactic-co-glycolic acid) (PLGA)-polyethylene glycol (PEG) copolymer has been selected for the development of polymeric NPs. PLGA is a biocompatible and biodegradable polymer composed by of glycolic and lactic acid monomers linked by ester bonds [1]. It is approved by both the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for use in drug delivery systems for parenteral administration, and has been widely explored in the design of NPs for drug delivery purposes in a wide range of biomedical applications [2]. PLGA has also been proposed for the development of nanotechnology-based microbicides [3]. PEG is also a extensively used polymer in the biomedical field, particularly for conferring improved solubility, stealth-like behavior (i.e., decreased uptake by the reticuloendothelial system) and non-immunogenic properties to nanosystems [4]. This polymer has further been proposed for densely coating microbicide nanosystems, providing these the ability to reduce interactions with mucins and favor their distribution and transport across mucus [5], as well as the ability to avoid liposomal uptake. For these reasons, PLGA-PEG copolymer (**Figure 1.1**) has been chosen as the building block for the polymeric NPs developed herein.

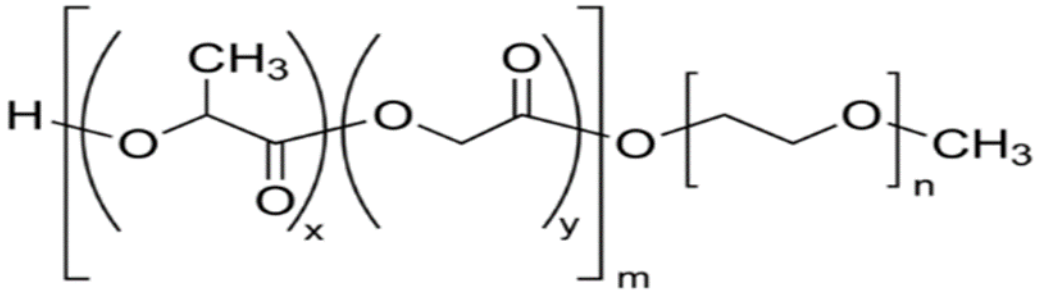

Figure 1.1 Structure of the PLGA-PEG polymer used in this work.

Regarding the ability to silence CCR5 expression, there are several siRNAs, that differ in the nucleotide sequence, commercially available. One of the strategies that has been used to increase the association efficiency between siRNAs and polymeric nanoparticles is the pre-complexation of the nucleic acid with different polyamines [6]. Two of those, spermidine and putrescine, were selected to improve the association of siRNA within the PLGA-PEG NPs in this work.

Specific aims of the work included the screening of several siRNAs with different transfection agents, in order to compare the ability of commercialized transfection agents and the produced PLGA-PEG NPs to deliver the siRNA and, ultimately, silence CCR5 expression.

1.2 Document Structure

This dissertation is organized in five chapters. Chapter 1 explores the rationale behind the present work and delineates its main objective. In Chapter 2, the most relevant literature is revised, describing the state of the art of research involving RNAi approaches for HIV prevention, particularly focusing on delivery systems. Chapter 3 is dedicated to the description of materials, experiments and methodologies used, while Chapter 4 presents the results and their critical and concise analysis. Finally, the main findings of this work are summarized and prospects for future studies are presented in the final chapter.

CHAPTER 2

Literature Review

2.1 Introduction

Acquired immune deficiency syndrome (AIDS), consequence of the infection by human immunodeficiency virus (HIV), is still one of the biggest healthcare issues worldwide, being estimated that more than 35 million people are infected. Incidence wise, close to 2 million people were infected during 2017 alone [7]. For both these statistics, more than two thirds of the cases were registered in Africa, particularly sub-Saharan countries [7], a region known for its countries', except South Africa, generally extreme poverty. The major route of HIV propagation, responsible for about 80% of the total number of infections, is sexual transmission [8]. Several and diverse approaches to treat this infection have been idealized and tested. One of the major strategies consists of antiretroviral therapy, that, despite significantly improving HIV infected patients' life quality and expectancy [9, 10] and preventing new infections [11], presents some shortcomings related with side effects of used drugs, namely cognitive impairment, due to neural toxicity [12], immune and renal dysfunction [13] and neural toxicity [14]. Thus, a highly effective and safe therapeutic option is still at large. Regarding infection prevention, a wide range of measures, such as condom usage, male circumcision and oral pre-exposure prophylaxis (PrEP), have been proven to greatly reduce the risk of sexual transmission [15]. However, each of these presents limiting factors. Condom usage is still associated with social stigma, and religious and moral beliefs also hinder its usage, particularly in less developed countries [16]. The main drawback regarding male circumcision is related to the fact that it is not clear that the protective effect is extended to sexual partners [17, 18]. Finally, lack of adherence to the regimens, as well as kidney and liver damage, represent the major handicaps of oral PrEP [19]. These do not allow the mentioned approaches to be fully effective, leading to a decrease in newly infected people slower than desired. Thus, development of new approaches is fundamental in order to better tackle this issue.

Alongside oral PrEP, topical approaches encompassing the use of microbicides have been developed and tested for blocking the virus transmission at rectal or vaginal mucosae [20, 21]. The first microbicides to be developed consisted of vaginal gels based on compounds that had shown activity against the virus *in vitro*. One category of those are surfactants, substances that disrupt viral membranes in non-specific fashion. While several surfactants have shown promising results, not a single one achieved overall positive outcomes, having failed at some point during clinical trials [22-25]. Several other strategies were followed, aiming at one of two outcomes, either blocking the viral entry in the host cells or targeting viral factors, preventing the virus from replicating and spreading the infection. Regarding the former, the most notable drug developed was maraviroc, a blocker of CCR5, one of the co-receptors used by the virus to enter the cells. Despite indications that this compound effectively blocked viral entry in CCR5 positive cells [26, 27], the concentration of maraviroc observed in macaques was not high enough to offer protection during a limited period of time [28, 29]. On the other hand, dapivirine [30-32] and tenofovir [33, 34] also found great success being administered as antiretroviral drugs. A different approach that has also been receiving wide consideration is the use of antibodies, mostly broadly neutralizing antibodies (bNAbs), in order to prevent the infection [35, 36]. Particularly, antibodies targeting the viral envelope, especially the gp120 protein have been extensively researched [37, 38]. Despite promising results, the HIV ability to deeply and quickly mutate, which can lead to viral escape, is still a major hurdle to overcome regarding this approach [39].

Thus, despite relative success, several limiting factors have hampered the achievement of greater outcomes. Some of these include the difficulty in scaling-up production, stability under different environmental conditions and safety [40]. Moreover, since the greatest challenge regarding AIDS eradication concerns sub-Saharan Africa, low cost of manufacture and ease of use are also essential conditions.

Following this direction, nanotechnology-based microbicides have been gaining interest recently [3]. Nanoparticles' properties, such as their size, large surface area and ease of functionalization make them a very promising approach for a wide range of biomedical fields, including HIV prevention [41]. While being developed to use alongside with more traditional antiretroviral drugs and monoclonal antibodies, nano-microbicides allow the exploration of alternative approaches. In particular, the use of gene editing and silencing is a strategy that has been receiving a lot of focus [42-44].

DNA editing approaches face a major obstacle: it may not be appropriate for use in healthy individuals. There are, although, promising approaches, such as the use of the CRISPR/Cas9 technology [45, 46]. On the other hand, gene silencing can also be attained by RNA interference (RNAi), a biological process that is triggered by short double-stranded RNA sequences, and which can be considered one of the most recent approaches for preventing HIV infections [47-49]. This effect can be induced either by endogenous small RNAs, named microRNAs (miRNAs),

by chemically synthetized exogenous small interfering RNAs (siRNAs) or by vector based short hairpin RNAs (shRNA). Despite their variable origin, the mechanism of action is similar. One of the strands is degraded upon binding to the RNA induced silencing complex (RISC), while the other strand leads the complex to degrade the corresponding mRNA [50]. Each of these approaches for gene silencing has its advantages and drawbacks, that will be latter further explored. It is broadly accepted that RNAi is an extremely attractive strategy to further research regarding HIV prevention. While several RNAi approaches regarding HIV have been showing positive indications, successful delivery is still one of the major obstacles [51-53]. Nanocarriers present the potential to surpass this hurdle, hence being a major focus of attention for RNAi delivery. Overall, it is vital that these strategies improve safety and efficacy profiles, and, when used to target viral moieties, are able to overcome the high mutability presented by HIV, since it is the major hurdle regarding the prevention of this infection.

2.2 HIV infection and possible targets for prevention

HIV infection and replication cycle are well established (**Figure 2.1**). This virus infects different cells of the immune system, mainly CD4-expressing T lymphocytes, monocytes and macrophages, but also dendritic cells. To do so, the virus fuses its viral envelope with the cell membrane [54]. The host receptor essential for this fusion to happen is CD4, which binds with high affinity to gp120, a family of glycoproteins existent on the HIV viral envelope. As consequence of this binding, gp120 suffers a conformational change, freeing a site that binds to a host co-receptor at the outer cell membrane. In the majority of the cases, this co-receptor is either CXCR4 or CCR5, being the latter overwhelmingly used by the virus in the infection initial phase [54]. After internalization of its core content, and using its own reverse transcriptase, the viral RNA is converted into double stranded DNA, which is then transported to the nucleus and incorporated in the genome by viral integrases. The expression of the viral DNA results in mRNAs that can be differently spliced, resulting in over 40 different mRNA species. Double-spliced fragments encode the proteins Tat, Nef and Rev [55]. This last one will accumulate, facilitating and mediating the nuclear export of the unspliced fragment, that encodes the Gag and Gag-Pol proteins, and the single-spliced fragments, which express Vif, Vpr, and Env proteins [56]. Inhibiting one or more of these viral proteins is a potential preventive approach that has been explored and will be further discussed. Moreover, since endogenous machinery is subverted and used for these processes, host factors are also an attractive preventive target, particularly during the earlier stages of infection.

One of the main characteristics that make HIV such an elusive virus is its ability to mutate itself, dodging host defense mechanisms. The first occurrence of this genetic plasticity is shown in the viral envelope, a structure that constantly changes and promotes direct consequences

regarding viral tropism, and preference to either one of the mentioned co-receptors. For instance, both in the early and chronic stages of the infection, CCR5-using viruses are dominant [54].

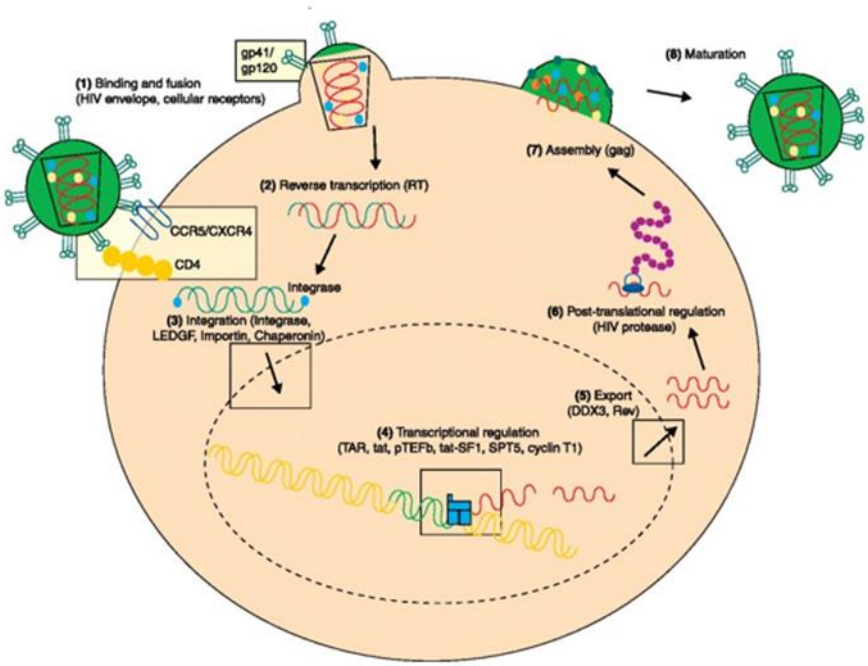

Figure 2.1 Several steps of the HIV-1 viral replication cycle and possible RNAi targets. Adapted from [47], Copyright © Bobbin et al.; licensee BioMed Central. 2015.

2.3 shRNA vs siRNA vs miRNA

RNAi is a mechanism that can be induced both endogenously and exogenously. In the first case, miRNAs are produced by the cell with the purpose of binding to mRNAs, making it possible to control several cellular processes through translational repression of the referred mRNA. In order to do so, the miRNA binds to a protein complex, RISC, that mediates the process [57]. The existence of this RNAi machinery turned out to be a very attractive target for specific gene silencing. There are two different strategies to achieve this, each with its advantages and drawbacks.

2.3.1 shRNA

For shRNA to be internalized by the cell, a non-replicating viral vector is commonly used. This allows an easier delivery of the shRNA into the nucleus, leading to its incorporation in the host genome, which, by its turn, leads to a stable integration and long-term expression of the incorporated nucleic acid [58]. Since shRNAs are designed to be processed as closely as possible

to the endogenous miRNAs [50], it is possible that it leads to toxicity, by competing with those host miRNAs and saturating the RNAi machinery [59]. One strategy to avoid this is the use of miRNA-based vectors for shRNA expression [60]. Beyond improving safety, this approach does not just increase the shRNA expression, as it allows the expression of multiples shRNAs from the same mRNA backbone. Since one of the major issues regarding HIV prevention is the ability of the virus to mutate and escape targeted therapies, this represents a major breakthrough [59]. Another advantage relies on the fact that traditional constructs require the integration of more than one copy in the host genome, while miRNA-based constructs can silence a gene with a single copy [61]. One of the biggest disadvantages of this technology resides in the fact that chemical modification of the shRNA is incredibly limited [50]. Moreover, targeting multiple sequences, while feasible, is slow and expensive. Since this approach has as goal the incorporation in the genome, long term safety and control might also be potential issues [57].

2.3.2 siRNA

For short term gene knockdown, i.e., transient RNAi effect, siRNA transfection is a more attractive approach. The better understanding of the endogenous RNAi pathway allows improved rational for the design of synthetic siRNAs. Because of its synthetic origin, it is possible to extensively modify its chemical properties in order to modulate its specificity and stability [57]. Effective delivery methods are essential for the development of this technology, since siRNA is negatively charged and susceptible to endogenous nucleases. Delivery approaches that might help surpass this problem will be discussed further on.

2.3.3 miRNA

While sharing several similarities with siRNAs, miRNAs differ in a significant aspect. While siRNAs possess a high specificity for its target, by aiming to silencing only one gene, miRNAs have the ability of targeting multiple genes [62, 63]. The complexity inherent to this multiplicity of targets, lead, immediately after the discovery of these mechanisms, to a stronger focus of research on siRNAs rather than miRNAs. However, with the recent attention given to miRNAs in research concerning RNAi pathways, significant advancements can be expected [62].

Besides endogenous miRNA, several viruses also produce their own miRNA, being actively involved in the major virus processes, such as replication and survival [63, 64]. However, regarding HIV, there is controversy regarding its ability to produce miRNAs [65]. RNA viruses are generally regarded as not producing miRNA [66], mainly because they are not located in the nucleus, thus not having access to the necessary machinery [67]. Nevertheless, HIV, being

a retrovirus, has its RNA transcribed into double stranded DNA, which is incorporated in the genome, providing the virus with access to nuclear machinery involved in miRNA pathways. Assuming that HIV does indeed produce miRNAs, it can represent an attractive target to prevent the infection, by subverting the viral machinery, in a similar fashion to what the virus does with the host machinery.

2.4 siRNA approaches for the prevention of HIV infection

As mentioned above, siRNA approaches may target both viral and host factors that play a role in HIV infection.

2.4.1 Viral factors

Since the life cycle of HIV is well known, there are several factors that can be targeted and inhibited in order to prevent the infection. Moreover, the fact that viral proteins are produced from a single mRNA, that is later cleaved, allows for the targeting of any region of the full-length mRNA as an approach to prevent the infection. Several viral mRNAs have been targeted in order to achieve that.

One of those is *tat*, a transcript that encodes a protein that is responsible for the regulation of viral gene expression, by stimulating transcriptional elongation [68]. Interestingly, it has been reported that the protein Tat is also responsible for suppressing a virus-specific siRNA precursor coded by HIV itself [69]. This viral factor is, therefore, a very appealing target. *Tat* transcript has been silenced by different siRNAs, using mostly lentiviral-based vectors as a delivery method [70-72].

Other potential targets for siRNA are the *env* regions of the viral genome. The protein encoded by this mRNA, Env, plays a major role in the attachment of HIV to target cells, as well as in the membranes fusion process [73]. It has been shown that siRNA successfully targeting and silencing these genes leads to the inhibition of HIV replication in peripheral blood mononuclear cells [74]. One of the systems targeting this gene uses a lentiviral vector as a delivery system [75].

The *gag* gene encodes a polyprotein that is cleaved into four different polypeptides, p17, p24, p7 and p6. The latter two are believed to be involved in the viral RNA binding, while p17 is a matrix protein and p24 a component of the capsid [76]. Is has been shown that targeting

the *gag* region of the viral genome leads to decrease in viral activity [77], including in CD4+ cells [78].

Unlike *gag*, *nef* is a non-structural gene. It is expressed before the virus integrates itself into the host genome [79]. It was demonstrated that *nef* inhibition by siRNA leads to a decrease in the expression of the viral transcript and confers some degree of resistance to HIV replication [80].

Yet another viral factor that might be a potential target for siRNA silencing is the gene *rev*. The expression of Rev protein leads to the cytoplasmic expression of the mRNA responsible for the expression of viral proteins previously mentioned, such as Gag and Env [81]. It has been shown that siRNA specifically targeting the *env* gene has the ability to substantially decrease viral activity in human cells [82].

While the mentioned are the HIV transcripts receiving more attention regarding siRNA approaches, there are other that have been targeted by siRNA, such as *pol* [83] or *vif* [71, 84].

2.4.2 Host factors

One of the major hurdles regarding the targeting of viral factors is the ability of HIV to mutate itself, which arises from the highly erroneous viral reverse transcriptase, and allows the virus to avoid silencing. Moreover, the silencing of viral structures is more promising regarding HIV treatment rather than prevention. Thus, targeting host factors, particularly those that are involved in pre-integration steps of the infection, seems like a very attractive alternative approach for HIV prevention.

The most obvious of these factors is CD4, since it is the receptor responsible for HIV recognition and binding to target cells. While it has been shown that silencing the expression of CD4 does indeed inhibit the HIV infection [85, 86], it is likely that, due to its particularly important role in immune processes, namely T CD4+ cells activation, the suppression of this receptor can lead to immunodeficiency [48].

One alternative that has been explored is the suppression of the co-receptor CXCR4. This is the co-receptor used by T cell tropic HIV strains [54]. It has been shown that siRNA targeting this protein leads to downregulation of CXCR4, as well as a lower level of transcripts, in both primary peripheral blood mononuclear and T cells [87], as well as Magi-CXCR4 cells [88]. Unlike CD4, CXCR4 depletion was found to not have a major impact in T cell maturation [89]. However, it has an important role in the trafficking of these cells, being pivotal for B cells, cardiovascular and cerebellar development [90]. Furthermore, this co-receptor is most notably used by viruses

in later stages of the infection rather than at transmission, making it less attractive from a prevention perspective [91].

The other co-receptor is CCR5, used by macrophage tropic strains of the virus [92], which is considered to be the most attractive target for RNAi approaches. One of the most, if not the most, important reasons for that is the related to a CCR5 mutation, known as CCR5Δ32. This mutation, discovered in 1996, consists in the deletion of 32 base pairs from the DNA sequence, leading to a frameshift at the amino acid level, and, consequently, rendering the co-receptor non-functional [93]. Since CCR5 is essential for HIV-1 entry in the cells, namely at sexual transmission, this mutation confers protection from viral infection. Thus, CCR5 may be regarded as the most attractive host target for HIV prevention, in particular through gene editing or silencing approaches. It has been shown, both *in vitro* [71, 94-96] and *in vivo* [97-100], that RNAi used in order to silence the expression of this co-receptor, particularly siRNAs, confers resistance to HIV-1 infection.

A slightly different approach that has been also pursued consists in targeting multiple factors, both viral and from the host [71, 85, 101]. While it seems promising and attractive, unintended effects of targeting host factors other than CCR5 and the high mutability evidenced by the virus might indicate that targeting CCR5 is a safer and more sustainable approach for HIV prevention through RNAi.

2.5 Delivery systems

In order for siRNA approaches to be effective, optimized delivery systems need to be developed. These can be separated in two major groups: viral and non-viral vectors.

2.5.1 Viral vectors

The initial approach to deliver nucleic acids in general, and siRNA in particular, was to use viral systems. These vectors are particularly attractive due to their high efficiency. However, concerns were quickly raised, mainly regarding safety hazards, such as immunogenic effects and the potential to trigger oncogenic responses [102]. Viral vectors can be further divided in different subsets, accordingly to which type of virus is used as a carrier.

One of the most explored groups of viruses for siRNA delivery has been lentivirus. These viruses have the ability to stably express siRNA or shRNA. Moreover, these vectors can transduce non-dividing cells, as they are able to target the nucleus. Furthermore, they are likely to be less immunogenic when compared with other viral vectors [103]. Adding the fact that have been used in several types of cells [84, 88, 104], the use of lentivirus can be regarded as one of the best approaches for viral delivery of siRNA.

Adenoviruses are also widely used regarding gene silencing. In most cases, when this type of viral vector is used, a double stranded DNA (dsDNA) construct is delivered into the cells, posteriorly expressing the correspondent siRNA [105-107]. The major distinctive factor when compared with lentiviruses is the fact that with the former, the siRNA is not integrated in the chromosomal DNA, limiting the use of this type of virus to situations where transient silencing is desired, or when the cells to transfect either replicate very slowly or are difficult to transfect [108]. On the other hand, with these vectors, insertional mutagenesis is not a concern, while high rates of transfection are achieved.

Similar to adenoviruses, adeno-associated viruses (AAV) are also used for transient transfections. However, there are three main differences between these two types of vectors. AAV have all of the viral genes removed, whereas some adenoviruses still express native genes, which leads to higher risk of immunogenic response. The other differences regard the packaging capacity, which is substantially higher in adenovirus, and the fact that AAV vectors are usually used for single stranded DNA (ssDNA) [107].

Retroviruses other than lentiviruses, commonly referred to as standard retroviruses, have also been used for attainment of RNAi regarding HIV infection. This type of vector has been shown to be able to successfully deliver shRNA, which is then processed into siRNA, to otherwise difficult to transfect cells [109, 110]. These viral vectors are often associated with immune responses, due to their integration. Overall, these retroviruses have been replaced by lentiviruses, due to higher efficiency and safety from the latter [111].

2.5.2 Non-viral vectors

While virus mediated delivery/induction of siRNA was relatively successful, the underlying potential of immunogenic and oncogenic triggers lead to a shift of research and development towards non-viral vectors [52].

This includes lipid-based delivery systems. The rationale behind these systems relies on the electrostatic interaction between the positively charged lipids and the negatively charged siRNA. Furthermore, the cationic nature of the complexes that are formed allows an easier cellular uptake of the siRNA [112]. Lipid-base delivery systems, such as liposomes, have been shown to successfully deliver siRNA to mucosal cells upon intravaginal administration [113], targeting both viral and host factors [114]. However, some lipid agents might show some level of toxicity themselves, possibly related to a boost, in dose-dependent fashion, of the viral infection. Macromolecules, such as cholesterol, have also been tested as a carrier for siRNA in the vaginal tract. While the issues observed with liposomes and other lipid-based systems were not registered, inconclusive and contrasting results regarding the protective effect of this formulation were observed [114, 115].

Polymeric nanoparticles have also been studied for intravaginal delivery of siRNA [6, 115, 116]. The main advantages of such delivery systems rely on the ability to protect the siRNA from being degraded, to control the rate of siRNA release, to penetrate tissues and to allow targeted delivery [51]. While non-biodegradable polymers such as polystyrene have been studied [117], the most used polymer for this purpose is very likely poly(lactic-*co*-glycolic acid) (PLGA) [6, 118, 119]. One of the most attractive properties of this polymer relies on its biodegradability, which does not only improve safety profiles, but also allows the release to be controlled and easily tuned. PLGA has been shown, both by itself [6] and in conjugation with other polymers [120], to effectively deliver siRNA when administered intravaginally. However, the anionic acid groups present in this polymer cause electrostatic repulsion when in the presence of siRNA phosphate groups. Furthermore, since siRNA has relatively low molecular weight and is hydrophilic, its association efficiency to PLGA nanoparticles is relatively low [121]. In order to circumvent this issue, pre-complexing siRNA with other molecules can be performed, in order to obtain complexes with high molecular weight and lower aqueous solubility, allowing for a more efficient association to nanoparticles [6, 122]. Another issue facing PLGA nanoparticles is the low ability of this system to penetrate the mucus layer, condition fundamental for siRNA delivery [123]. One approach widely used to increase mucus-penetrating properties of several carriers, from polymeric nanoparticles to lipid-based vehicles, is dense surface modification with polyethylene glycol (PEG) of relatively low molecular weight (typically 5-10 kDa) [121]. Using this polymer also brings the advantage of promoting lysosomal escape [124]. Polymeric nanoparticles seem, then, to be an attractive approach for topical siRNA delivery to prevent HIV infection by the vaginal and rectal routes.

Even though mild success has been obtained with traditional microbicides, such as the dapivirine ring and one tenofovir gel, the broader potential of gene silencing, namely concerning siRNA, makes it an interesting preventive avenue to pursue. Despite being extensively studied, and with relative success, delivery through viral vectors and targeting of viral factors raise some concerns regarding, respectively, safety and efficacy. Thus, a shift towards non-viral vectors and targets for gene silencing has been occurring. However, even if safety and efficacy are demonstrated, the development and successful attainment of these potential solutions still face serious challenges, such as the ease of scaling-up production and the final cost of the solutions.

CHAPTER 3

Materials and Methods

3.1 Materials, reagents and cell lines

Poly(ethylene glycol) methyl ether-block-poly(lactide-*co*-glycolide) (PLGA-PEG; 50:50 lactide:glycolide molar ratio; PLGA number average molecular weight (Mn) 55 kDa; PEG Mn 5 kDa) was purchased from Sigma Aldrich (St. Louis, MO, USA). Kolliphor® P407 was purchased from BASF (Ludwigshafen am Rhein, Germany). Ethyl acetate and dimethyl sulfoxide were obtained from Merck Millipore (Billerica, MA, USA). Two different CCR-5 specific siRNAs were purchased from either Santa Cruz Biotechnology (Dallas, TX, USA) (from now on siRNA (SCB)) or Thermo Fisher Scientific (Waltham, MA, USA) (siRNA (TFS)). A non-silencing siRNA was also obtained from Santa Cruz Biotechnology. Nuclease-free water and Opti-MEM Reduced Serum Media were both purchased from Thermo Fisher Scientific. ScreenFect Enhancer was purchased from ScreenFect GmbH (Eggenstein-Leopoldshafen, Germany). Polyethyleneimine (PEI, branched, M.W. 10,000, 99%) was obtained from Alfa Aesar (Haverhill, MA, USA). Lipofectamine RNAiMAX Transfection Reagent was acquired from Thermo Fisher Scientific. Allophycocyanin (APC)-labelled anti-CCR5 antibody [CD195 (CCR5) Monoclonal Antibody (NP-6G4), APC, eBioscience™] was obtained from Invitrogen (Carlsbad, CA, USA). Maraviroc was extracted and purified from Selzentry® tablets (ViiV Healthcare, Algés, Portugal) following a previously reported method [125]. Spermidine and putrescine were purchased from Sigma Aldrich. SYBR™ Gold Nucleic Acid Stain (10,000 X Concentrate in DMSO) was acquired from Invitrogen. Ghost (3) $CCR5^+$ cells (Hi-5 cells; CCR5-expressing cells originated from human osteosarcoma) available from the NIH Aids Reagent Program were kindly provided by Prof. Nuno Taveira, iMed.ULisboa, University of Lisbon. Caco-2 (C2BBe1 clone) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and 0.25% (w/v) trypsin-0.53mM EDTA were purchased from Invitrogen. All solvents and reagents were of analytical grade or equivalent.

3.2 Production and characterization of nanoparticles

3.2.1 Production of nanoparticles

PLGA-PEG NPs were produced by a double emulsion solvent evaporation technique, as depicted in **Figure 3.1**. Briefly, 20 mg of copolymer were dissolved in 1 mL of ethyl acetate, and 250 µL of water (internal phase) were added and the mixture vortexed (VV3 vortex, from VWR (Radnor, PA, USA)) at full speed during 30 seconds in order to form a W/O primary emulsion. Then, 5 mL of either poloxamer 407 at 0.2% (w/v) or nuclease-free water was added, and the mixture was sonicated (VibraCell VC50, with stepped microtip (2mm), from Sonics & Materials (Newtown, CT, USA)) for 60 seconds, at 70% intensity, in order to form a W/O/W secondary emulsion. Finally, the multiple emulsion was further diluted with 10 mL of either poloxamer 407 at 0.2% (w/v) or nuclease-free water and left under magnetic stirring (300rpm) for 4 hours, in order to allow partial evaporation of ethyl acetate and formation of solid nanoparticles. Nanoparticles were then concentrated and washed three times with 10 mL of nuclease-free water by centrifugation at 2,100 rpm for 12 minutes (Eppendorf 5810R Refrigerated Centrifuge) using Amicon Ultra-15 Centrifugal Filter Units (Millipore) with a molecular weight cut-off (MWCO) of 100 kDa. The volume of nanoparticle suspensions was adjusted to 1.5-2 mL with nuclease-free water and kept at 2-8 °C until further use. To lyophilize the nanoparticles, the suspension was frozen at -80 °C for at least 4 hours. Frozen nanosuspensions were then freeze-dried at -80 °C and at pressure equal to or lower than 0.008mbar (FreeZone Plus 2.5 Liter Cascade Console, from Labconco (Kansas City, MO, USA)).

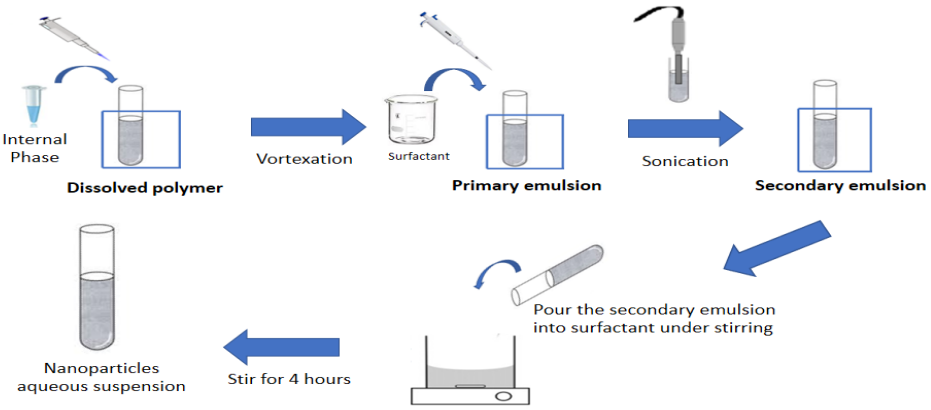

Figure 3.1 Schematic representation of the double emulsion solvent evaporation technique used for the production of polymeric nanoparticles. Please see main text for details.

When required, siRNA was incorporated into nanoparticles by pre-complexing it with either spermidine or putrescine, at a nitrogen/phosphate (N/P) ratio of either 8/1 or 3/1, and included in the inner aqueous phase before nanoparticle production. The amount of each polyamine to be used in order to achieve these ratios was calculated as previously described [6, 126]. For complexation, siRNA and the respective polyamine were diluted in nuclease-free water to a final volume of 125 µL each. The diluted siRNA was then added to the diluted polyamine, and the mixture was incubated at room temperature for 20-25 minutes before proceeding with nanoparticle production.

Nanoparticles were produced using different compositions of the inner aqueous phase, as listed in **Table 3.1**.

Table 3.1 Different conditions used during the production protocol of NPs.

Designation	Internal Phase	siRNA content (pmole)	N/P ratio	n
NPs w/o surfactant	Water	-	-	1
Blank NPs	Water	-	-	7
l-siRNA NPs	siRNA	200	-	1
h-siRNA NPs	siRNA	1000	-	5
l-Spermidine 8/1 NPs	Spermidine-siRNA complexes	200	8/1	1
Putrescine 8/1 NPs	Putrescine-siRNA complexes	200	8/1	1
h-Spermidine 8/1 NPs	Spermidine-siRNA complexes	1000	8/1	5
Spermidine 3/1 NPs	Spermidine-siRNA complexes	1000	3/1	3

3.2.2 Nanoparticles characterization

Size (mean hydrodynamic diameter) and polydispersity index (PdI) of all NP formulations were determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK). The same equipment was used for assessing zeta potential of NPs by Electrophoretic Light Scattering (ELS). All assays were performed after 100 times dilution of NP dispersions with 10 mM sodium chloride solution. Three runs per sample were performed.

In order to quantify siRNA content, the total mass of lyophilized NPs was dissolved in 1 mL of ethyl acetate at room temperature for 2 hours. To extract siRNA, 500 µL of TE buffer (10mM

Tris containing 1 mM EDTA, at pH 7.4) were added to the previous, and the mixture was vortexed, at full speed, for 1 minute and centrifuged at 12,000 rpm and 4 °C for 5 minutes. This procedure was performed either once or twice, depending on the experiment. The aqueous fraction was retrieved, and siRNA content was assessed using SYBR™ Gold Nucleic Acid Stain. A standard curve correlating fluorescence and siRNA concentration was used to determine the amount of siRNA loaded into the particles. Briefly, the retrieved aqueous phase was diluted tenfold and 0.02µL of SYBR Gold were added. The obtained mixture was equilibrated for 10 minutes before proceeding with fluorescence readings at excitation and emission wavelengths of 495 and 540 nm, respectively, using a Synergy Mx fluorimeter and according to the instructions of the SYBR™ Gold Nucleic Acid Stain manufacturer.

3.3 Cell culture and maintenance

Ghost Hi-5 (passages 7-20) and Caco-2 clone cells (passages 78-80) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, and added with 100 U/mL penicillin and 100 µg/mL streptomycin (further referred as complete DMEM). Cells were kept in a cell culture incubator (Binder, Tuttlingen, Germany) at 37 °C and 5% CO2, under a water saturated atmosphere. Cells were sub-cultured every 3-4 days, using trypsin–EDTA as dissociation reagent.

3.4 *In vitro* cell studies

3.4.1 Transfections

Ghost Hi-5 cells were plated, unless otherwise stated, in 24-well plates at 1 x 10^5 at 24 hours before transfection. Two hours before transfection, medium was removed, and cells were washed twice with 500 µL of PBS (at pH 7.4). Plain medium (i.e., DMEM only) was added to each well. siRNA-transfection agent complexes were prepared by incubating different amounts of siRNA with several transfection agents (to be detailed further). The working solutions of siRNA and transfection agent were obtained by diluting stock solutions in Opti-MEM. Complexes, as well as the transfection agent alone, were added to cells to a final volume of 500µL. Cells were incubated at 37 °C and 5% CO2. Six hours post transfection, medium was removed and 1mL of DMEM supplemented with 10% FBS, but without antibiotics, in order to prevent cytotoxicity and improve the transfection's efficacy [127], were added. Cells were, unless stated otherwise, collected 72 hours post-transfection. Cells were detached using 500 µL of trypsin-EDTA and 1 mL of complete DMEM. The resulting 1.5 mL was transferred to Eppendorf tubes. Cells were then centrifuged at 4 °C, 300 xg, for 7 min (Eppendorf 5417R Refrigerated Centrifuge), the supernatants were discarded, and cells were resuspended in 100 µL of FACS buffer (PBS

containing 10% (v/v) FBS and 0.1% (w/v) sodium azide). Five microliters (0.25 µg) of the APC-labelled anti-CCR5 antibody were added to selected tubes and all samples were incubated for 45 minutes at 37 ˚C. Then, cells were washed by centrifugation (300 xg, 4˚C, 7 min), resuspended in 200 µL of FACS buffer. This washing procedure was repeated up to a total of three washes, unless stated otherwise. Cells were then transferred to FACS tubes and immediately analyzed by FACS using a BD FACSCanto II instrument (BD Biosciences, San Jose, CA, USA), equipped with three lasers: 405 nm, 488 nm and 633 nm. Upon excitation with the 633 nm laser, stained cells were detected through the APC channel. The results were analyzed using the FlowJo v10.0.7 software (FlowJo LLC, Ashland, OR, USA).

3.4.1.1 ScreenFect

Ghost Hi-5 cells were plated in 6-well plates at 5 x 10^5 cells/well at 24 hours before transfection. siRNA-transfection agent complexes were prepared by incubating different amounts of siRNA (1, 10 and 100 nM) with 4 µL of the transfection agent for 20 minutes at room temperature. A control non-silencing siRNA, at 100 nM, was also incubated with the same volume of transfection agent. Cells were collected 6, 12, 24 and 72 hours post-transfection. For this experiment, replicates were not performed. Moreover, only one step of washing, and not three, following antibody incubation was performed.

3.4.1.2 Polyethylenimine (PEI)

PEI transfection agent was prepared by diluting a viscous PEI solution in nuclease-free water to a concentration of 0.9 mg/mL, and posteriorly filtering through a 0.22 µm filter. siRNA-transfection agent complexes were prepared by incubating the same amount of siRNA (25 nM) with two different amounts of PEI, in order to obtain different nitrogen per phosphate (N/P) ratios. N/P ratios of 30 and 40 were used, corresponding to 3 and 4 µL, respectively, of the PEI solution. Posteriorly, different amounts of siRNA (25 and 50 nM) were complexed with PEI to obtain a N/P ratio of 40. siRNA-PEI complexes were obtained after 20 minutes of incubation at room temperature. In both experiments, a control siRNA was also incubated with the same volume of transfection agent. For the first experiment, all formulations were done in duplicates. For the latter, untreated and unstained cells were analyzed in duplicates, while the remaining conditions were performed in triplicates.

3.4.1.3 RNAiMAX

3.4.1.3.1 siRNA (SCB)

siRNA-transfection agent complexes were prepared by incubating varying amounts of siRNA with 1.5 µL of the transfection agent. Three experiments where performed with this siRNA and transfection agent. In the first, siRNA was used at 25 and 50 nM, while in the second, the concentrations used were 25, 50 and 100 nM. Finally, in the third experiment, siRNA was used at 100 and 200 nM. A control non-silencing siRNA was used in each experiment, at the highest concentration used for siRNA (50,100 and 200 nM, respectively), with the same volume of transfection agent. The complexes were left to incubate for 20 minutes at room temperature. For the first experience, untreated and unstained cells were performed in duplicates, and the other conditions without replicates. For the second experience, all the conditions were performed in duplicates. Finally, in the last experiment, cells treated with siRNA were done in triplicate, and the other conditions in duplicate.

3.4.1.3.2 siRNA (TFS)

siRNA-transfection agent complexes were prepared by incubating different amounts of siRNA (50 and 100 nM) with 1.5µL of the transfection agent. The complexes were left to incubate for 20 minutes at room temperature. Cells treated with siRNA were done in triplicate, while controls (unstained, untreated and treated with transfection agent only) were done in duplicate.

3.4.2 Maraviroc and Antibody testing

Ghost Hi-5 and Caco-2 cells were plated in 6-well plates at 5 x 10^5 cells/well and incubated at 37 °C and 5% CO2 for 24 hours. Maraviroc (MVC), previously dissolved in DMSO, was added to selected wells to a final concentration of 1 or 10 µM. All cells were incubated at 37 °C and 5% CO_2 for 1 hour, and then detached using 500 µL of trypsin-EDTA and 1 mL of complete DMEM. The resulting 1.5 mL was transferred to Eppendorf tubes. The cells were then centrifuged at 4 °C, 300 x*g*, for 7 min (Eppendorf 5417R Refrigerated Centrifuge), the supernatants were discarded, and cells were resuspended in 100 µL of FACS buffer. Five microliters (0.25 µg) of the APC-labelled anti-CCR5 antibody were added to selected tubes and all samples were incubated for 45 minutes at 37 °C. Then, cells were washed by centrifugation (300 x*g*, 4°C, 7 min), resuspended in 200 µL of FACS buffer and transferred to FACS tubes. The samples were immediately analyzed by FACS using a BD FACSCanto II instrument (BD Biosciences, San Jose, CA, USA), equipped with three lasers: 405 nm, 488 nm and 633 nm. Upon excitation with the 633 nm laser, stained cells were detected through the APC channel. The results were analyzed using the FlowJo v10.0.7 software.

3.4.3 NPs siRNA (TFS) delivery

Ghost Hi-5 cells were plated in 24-well plates at 1 x 10^5 cells/well 24 hours before transfection. Two hours before transfection, medium was removed, and cells were washed twice with 500 µL of PBS. Plain medium (i.e., DMEM only) was added to each well. Lyophilized nanoparticles (blank, h-siRNA, h-spermidine 8/1 and spermidine 3/1 NPs) were dispersed in DMEM only, to a concentration of 2 mg/mL. Dissolved nanoparticles were added to cells, at 0.1, 0.5, 1.0 and 2.0 mg/mL, corresponding to 5, 25, 50 and 100 nM of siRNA, respectively, to a final volume of 500 µL. Cells were incubated at 37 °C and 5% CO_2. Six hours post transfection, medium was removed and 1mL of DMEM supplemented with 10% FBS, but without antibiotics, was added. Cells were collected after 72 hours of incubation. The cells were detached using 500 µL of trypsin-EDTA and 1 mL of complete DMEM. The resulting 1.5 mL was transferred to Eppendorf tubes. The cells were then centrifuged at 4 °C, 300 x*g*, for 7 min (Eppendorf 5417R Refrigerated Centrifuge), the supernatants were discarded, and cells were resuspended in 100 µL of FACS buffer. Five microliters (0.25 µg) of the APC-labelled anti-CCR5 antibody were added to selected tubes and all samples were incubated for 45 minutes at 37 °C. Then, cells were washed by centrifugation (300 x*g*, 4°C, 7 min), resuspended in 200 µL of FACS buffer. This washing procedure was repeated up to a total of three washes. Cells were then transferred to FACS tubes. Then, samples were immediately analyzed by FACS using a BD FACSCanto II instrument (BD Biosciences, San Jose, CA, USA), equipped with three lasers: 405 nm, 488 nm and 633 nm. Upon excitation with the 633 nm laser, stained cells were detected through the APC channel. The results were analyzed using the FlowJo v10.0.7 software.

3.5 Statistical analysis

Results are presented as mean values ± standard deviation (SD). Statistical analyses were performed using unpaired t tests, unless otherwise stated. Differences were considered significant at $p<0.05$. Statistical significance was denoted if the p value was lower than 0.05 by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.005$). All analyses were performed with the GraphPad Prism 7.0 software.

CHAPTER 4

Results and Discussion

4.1. Ghost Hi-5 CCR5 expression

Transfection of Ghost Hi-5 cells with CCR5-specific siRNA was performed as extensively described. Complexes between siRNA and commercial transfection agents were prepared and delivered to cultured cells. CCR5 expression was assessed by FACS.

The population distribution and gating strategy adopted to perform the analysis are shown in **Figure 4.1**. The first panel shows the selection of the viable cells on a Forward Scatter Area (FSC-A) vs. Side Scatter Area (SSC-A) dot plot. The second panel shows the exclusion of cell doublets and aggregates, being only selected the single cells, on a FSC-A vs. Forward Scatter Height (FSC-H) dot plot. Finally, the last panel shows the signal of selected cells detected in the allophycocyanin (APC) channel (633nm). This sequential gating strategy allows the attainment of a more reliable signal, since both debris and dead cells are filtered out, as well as aggregates and doublets, whose signal could lead to misinterpretations.

In **Figure 4.2**, the histograms of the geometric mean of the fluorescence from the APC channel are presented. The first panel shows the histogram referent to a population of single cells, while in the second a comparison between the histograms of two different samples, also of single cells, is shown. In order to assess silencing effect, histograms of each sample were compared to the histograms of controls. Furthermore, the obtained geometric mean fluorescence values for each condition were also compared.

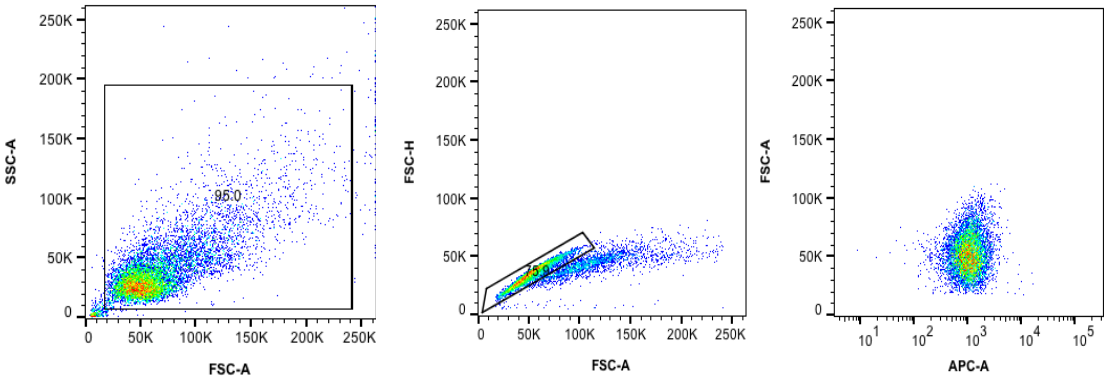

Figure 4.1 Representative population distribution and gates applied in FACS.

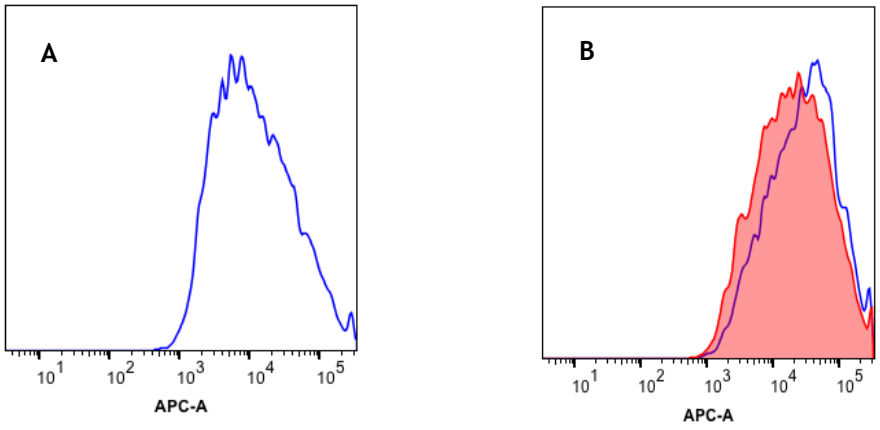

Figure 4.2 Geometric mean fluorescence histograms. (A) Fluorescence histogram of a single sample; (B) Comparison between histograms of a sample (blue line) and merged controls (red line, shaded).

4.1.1 ScreenFect Enhancer transfection

Initially, transfection efficacy using ScreenFect Enhancer as a delivery agent and siRNA (SCB) was tested. Three different siRNA concentrations (1, 10 and 100 nM) and four different time points (6, 12, 24 and 72h) were evaluated. The two first time points were chosen due to CCR5 turnover. In the absence of ligand, this receptor has a constitutive turnover with half-time between 6 and 9 hours [128]. On the other hand, the longer time points are both recurrently referred in literature for CCR5 knockdown [86, 95, 96]. After each time point, cells were collected and incubated with the APC-labeled CCR5 monoclonal antibody. Following incubation, cells were washed once, and then analyzed by flow cytometry. Geometric mean values obtained for each sample are shown in **Figure 4.3**.

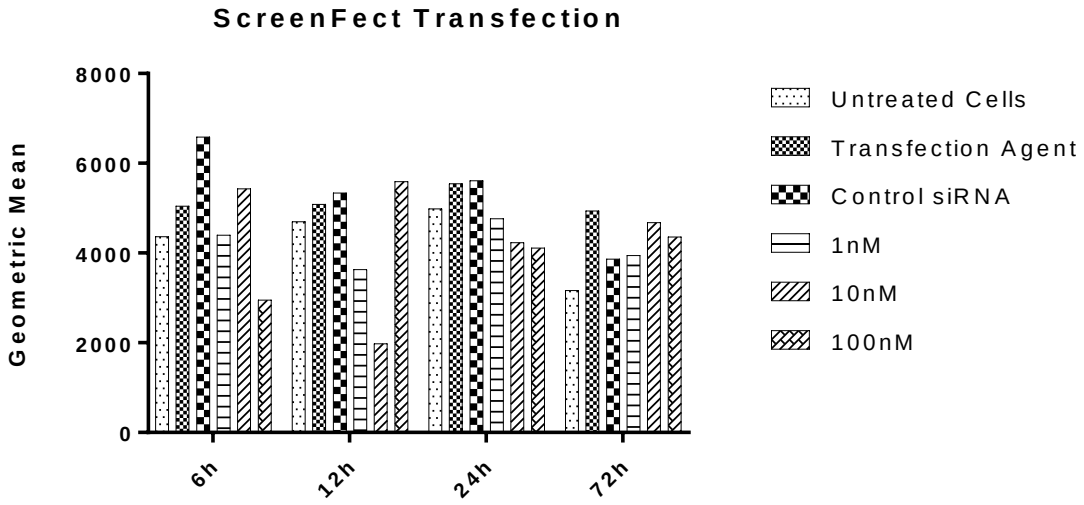

Figure 4.3 Quantitative expression of CCR5 by Ghost Hi-5 cells at different time points post transfection with ScreenFect Enhancer (n=1).

Although preliminary, the results regarding silencing did not seem to be consistent. For example, at 6 hours, CCR5 expression was lower in cells treated with 100 nM siRNA than in cells incubated with 10 nM siRNA. However, the opposite was observed for cells evaluated 12 hours after the transfection. On the other hand, at 24 hours, there seemed to be a slight decrease in CCR5 expression, for all concentrations tested, whilst at 72 hours there were no apparent changes between the samples and each of the three controls. Interestingly, for each of the time points analyzed, incubation with transfection reagent both alone and complexed with the control siRNA appears to have increased CCR5 expression.

Overall, and despite observing slight decrease in CCR5 expression for some time points and siRNA concentrations, particularly 12 hours post-transfection with 10 nM, and due to lack of replicates, it is impossible to conclude about the potency of the siRNA tested.

In order to assess the reasons for that, five scenarios were evaluated. The first was that the siRNA was not silencing the expression of the receptor properly. Other possibility was that the transfection agent used was not able to intracellularly deliver the siRNA to the cultured cells. Also, it was hypothesized that washing the cells only once after incubation with the antibody might have hindered the correct assessment of the transfection efficacy. Since it has been reported that several monoclonal anti-CCR5 antibodies being commercialized lack specificity [129], other potential issue regarding the assessment of the silencing could be related to the antibody used to quantify the receptor expression levels. Finally, it could be an issue regarding the phenotype of the cell line being used. Ghost Hi-5 cells were genetically modified from Ghost parental cells in order to over-express CCR5. In this case, it is possible that the silencing effect of the transfection could be overruled by the mentioned genetic modification, and a

higher percentage of silencing could not be achieved. However, it has been reported in similar cell lines that substantially stronger silencing of CCR5 is attainable [96]. Based on this, subsequent experiments were made in order to assess the reliability of the three reagents used (siRNA, transfection agent and antibody), as well as the implication of the number of washes following antibody incubation.

4.1.2 Caco-2 cells and Maraviroc

In order to assess the specificity of the antibody to CCR5, a cell line that does not express this receptor, Caco-2, was incubated with the antibody. Although the best control would have been Ghost parental cells, such cell line was not accessible in a timely manner. Furthermore, Ghost Hi-5 cells were also submitted to two different concentrations of maraviroc (MVC). MVC is known to block the interaction between the gp120 HIV protein and CCR5 by binding to the receptor and altering its morphology [130]. In order to be able to compare the data with the previously obtained results, cells were washed only once after incubation with the antibody. Results obtained are shown in **Figure 4.4**.

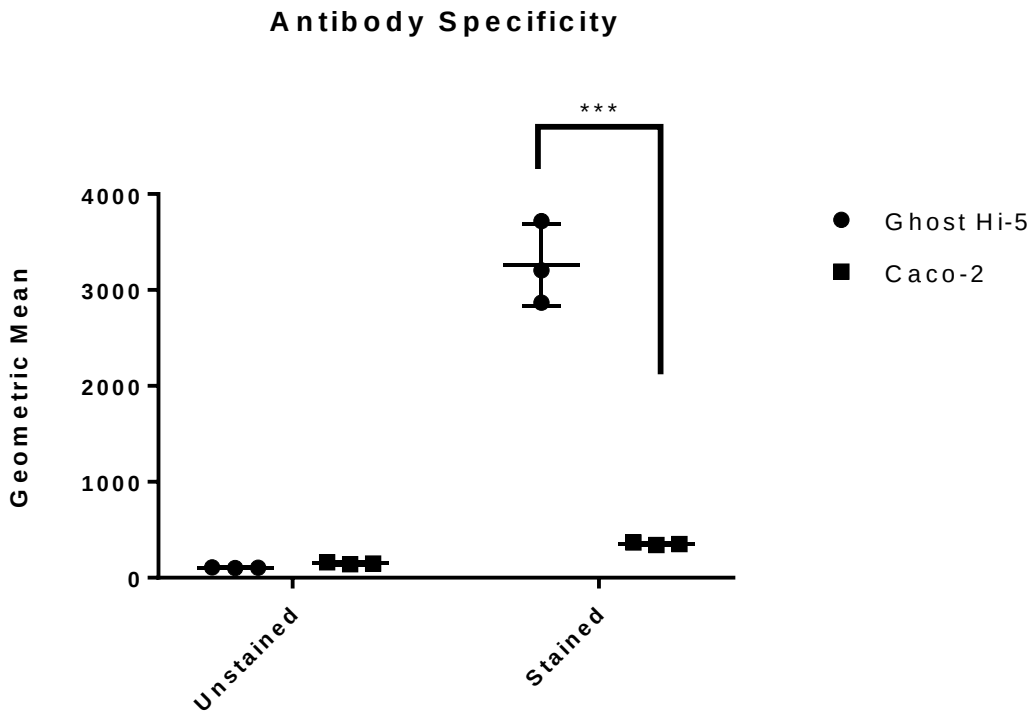

Figure 4.4 CCR5 expression by Ghost Hi-5 and Caco-2 cells. Results are presented as mean ± SD (n=3). (***) denotes a significant difference at p<0.005.

As clearly shown, the fluorescence values obtained for stained Ghost Hi-5 cells were significantly higher than those obtained for stained Caco-2 cells. This seems to indicate that

the antibody does indeed present binding specificity for CCR5. Moreover, the slight increase observed for Caco-2 cells incubated with the antibody, as compared to the same cells, appears to indicate that non-specific binding is quite low. It is also likely that such small difference is owed to only washing the cells once. Thus, in order to circumvent this issue in posterior experiences, three washes were performed after incubation with the antibody.

Regarding MVC, while there seemed to be a reduction in CCR5 expression, the data, shown in **Figure 4.5**, are not conclusive, particularly accounting for the lack of replicates of MVC treatments.

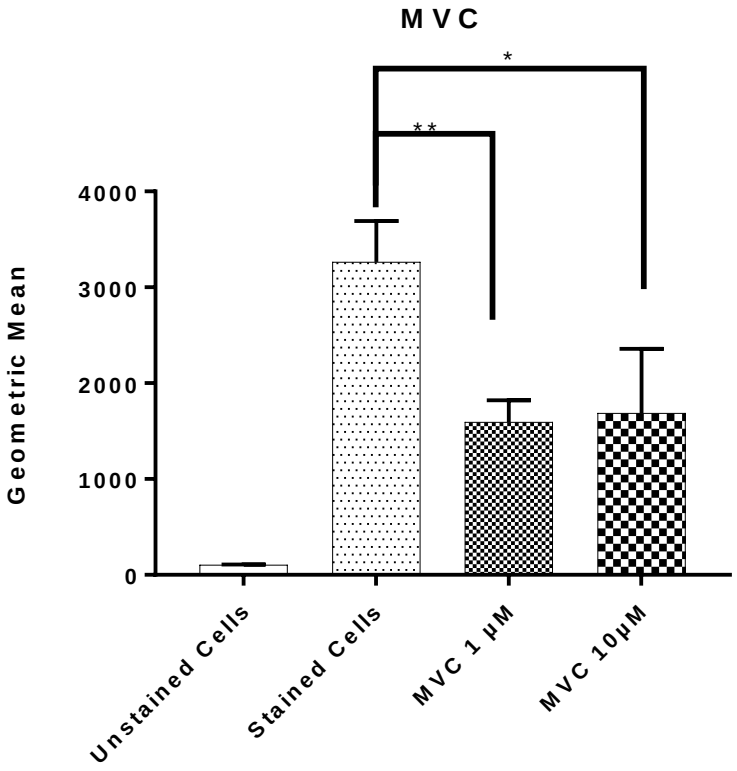

Figure 4.5 Quantitative CCR5 expression by Ghost Hi-5 cells after MVC treatment. Results are presented as mean ± SD (n=3). The replicates regard the antibody incubation, not the MVC treatment itself. (*) and (**) denote a significant difference at $p<0.05$ and $p<0.01$, respectively.

MVC is a small molecule and its binding to CCR5 may not be able to block all sites of the receptor that are recognized by the antibody. The mechanism of action of MVC consists in altering the conformation of CCR5 without inducing its internalization [131]. Mild reduction in CCR5 labeling in MVC-treated cells appears to support that interference with the receptor affects the binding ability of the antibody. However, it is wise to cautiously interpret these

results, since it has been described in literature that MVC does not alter [132] and, in particular conditions, such as in HIV-infected cells, even increases [133] CCR5 levels.

Since the antibody was apparently shown to properly and selectively bind to CCR5, the ability of different transfection agents to deliver the same siRNA (SCB) into Ghost Hi-5 cells and silence CCR5 was then tested. Also, from this point forward, after incubation with the antibody, cells were submitted to three washes rather than one. Moreover, in order to reduce the impact of the background values in the silencing assessment, settings of the data acquiring equipment, namely the voltage used to measure the fluorescence, were modified so that the values of that fluorescence were substantially higher. Since the increase was more pronounced for stained samples and controls than for unstained cells, the impact of background signal was reduced.

4.1.3 Polyethylenimine (PEI)

Polyethylenimine (PEI) has been shown able to mediate gene knockdown through RNAi when used as siRNA-PEI complexes [134, 135]. Two different siRNA concentrations (25 nM and 50 nM), as well as two different N/P ratios (30 and 40) were tested. While a wide range of ratios, ranging from 4 to 100, were analyzed in several contexts, high variability of the tested conditions, such as the molecular weight and the spatial organization of the polyamine (branched or linear) made it difficult to understand what ratios would be more indicated for this experiment. Based on literature that found a N/P ratio of 33 to be close to optimal [135], and in order to facilitate calculations, an approximate ratio of 30 was used. On the other hand, in a different study, the concentrations and quantities of both siRNA and PEI, rather than the N/P ratio, were taken into account [136]. Following this procedure, and calculating the ratio based on the same equations used previously [126], a N/P ratio of 40 was used. Comparisons between untreated and treated cells are presented in **Figures 4.6** and **4.7**.

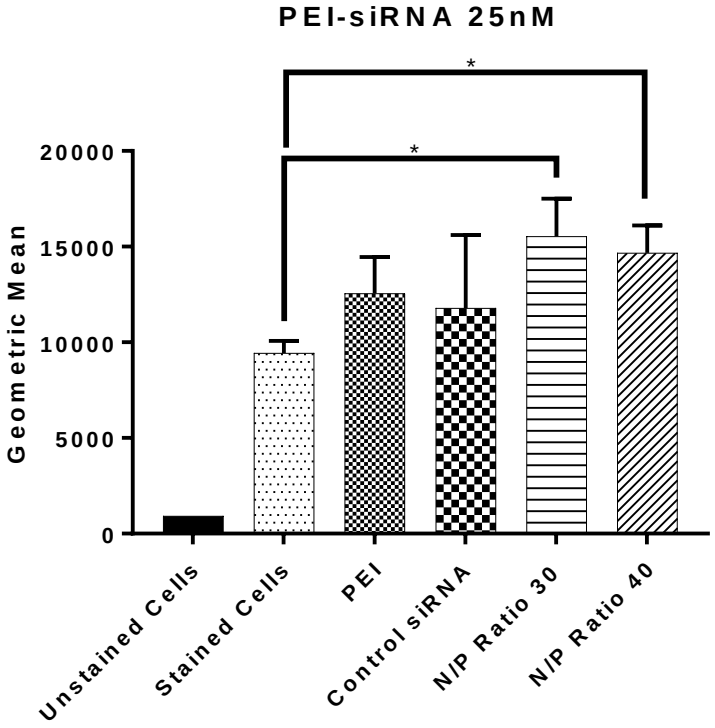

Figure 4.6 Quantitative CCR5 expression by Ghost Hi-5 cells 72 hours post transfection with PEI at different ratios. Results are presented as mean ± SD (n=3). (*) denotes a significant difference at p<0.05.

Interestingly, and in line with what was observed with the ScreenFect Enhancer transfection reagent, administrating PEI alone to the cells resulted in a considerable increase in CCR5 detection. Significant differences were obtained comparing untreated cells with both N/P ratios tested to deliver the siRNA. However, this difference corresponds to an increase in CCR5 expression, rather than silencing. Despite significance, all values are in the same order of magnitude and are, presumably, biologically irrelevant. Toxicity associated with PEI may also help explaining differences.

Since neither of the tested N/P ratios was able to provide a decrease in CCR5 expression, a higher concentration of siRNA, 50 nM, was further tested. In this case, the N/P ratio used was 40. Data obtained are shown in **Figure 4.7**.

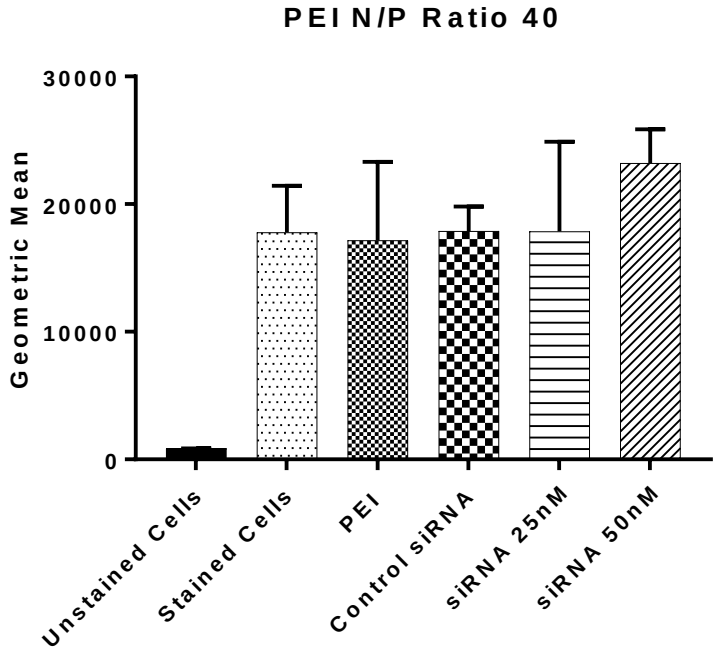

Figure 4.7 Quantitative CCR5 expression by Ghost Hi-5 cells 72 hours post transfection with PEI. Results are presented as mean ± SD (n=3).

There were no significant differences in the fluorescence levels obtained between each of the tested conditions. At this point, it seemed plausible to assume that siRNA (SCB) delivered through PEI complexation revealed itself not to work properly. In fact, cells transfected did not only shown no traces of silencing, as indeed they appeared to express CCR5 more strongly than untreated cells. Based on this, further clarification regarding the ability of this siRNA to effectively silence CCR5 expression was not attained.

4.1.4 RNAiMAX

Another transfection agent, RNAiMAX, was further tested. RNAiMAX is a commercially available reagent and is one of the most widely used transfecting agents [137]. In a first test, two concentrations of siRNA, without any replicates, were used with this carrier (**Figure 4.8**).

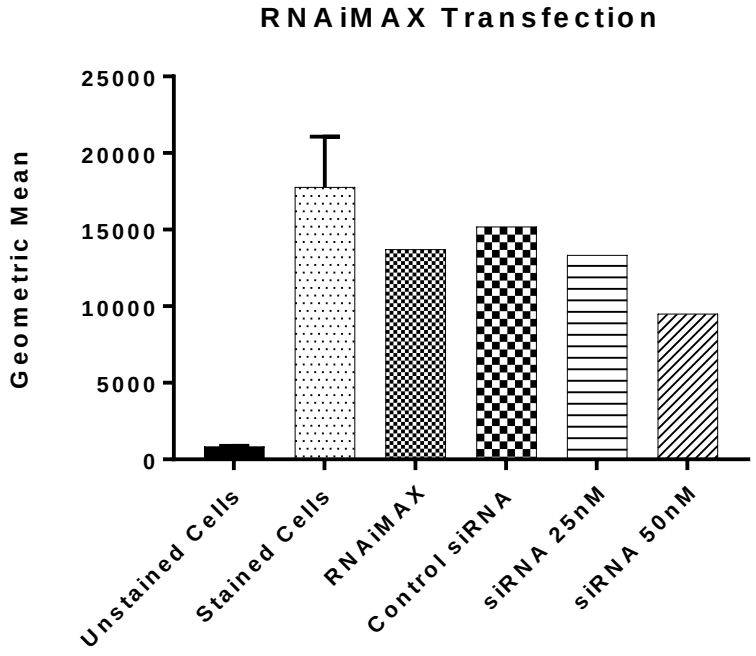

Figure 4.8 Quantitative CCR5 expression by Ghost Hi-5 cells 72 hours post transfection with RNAiMAX. Results are presented as mean ± SD (n=3 for unstained and stained cells, n=1 for other conditions).

Interestingly, unlike what was observed for previous transfection agents, RNAiMAX alone led to a slight decrease in CCR5 expression, as well as when complexed with the control siRNA. Off-target effects of transfection agents in general have been extensively reported [138-140]. RNAiMAX in particular has been found to increase mRNA levels of interest [141].

More importantly, it appears that siRNA at 25 nM does not lead to silencing effect when compared with the transfection agent alone. However, with 50 nM, there seems to be a slight decrease in the obtained fluorescence. Despite being lower than desired, and expected, according to literature [96], this modest evidence of silencing might indicate that, out of the three transfection agents tested, RNAiMAX is the one that more efficiently delivers the siRNA, thus leading to greater gene knockdown. It also allows the presume that, even if not working completely properly, this siRNA (SCB) may indeed have the ability to knockdown, at least to some extent, CCR5 expression. In order to obtain data to confirm this theory, several transfections using these same reagent and siRNA, at different concentrations, were performed. Data obtained from these transfections are shown in **Figures 4.9** and **4.10**.

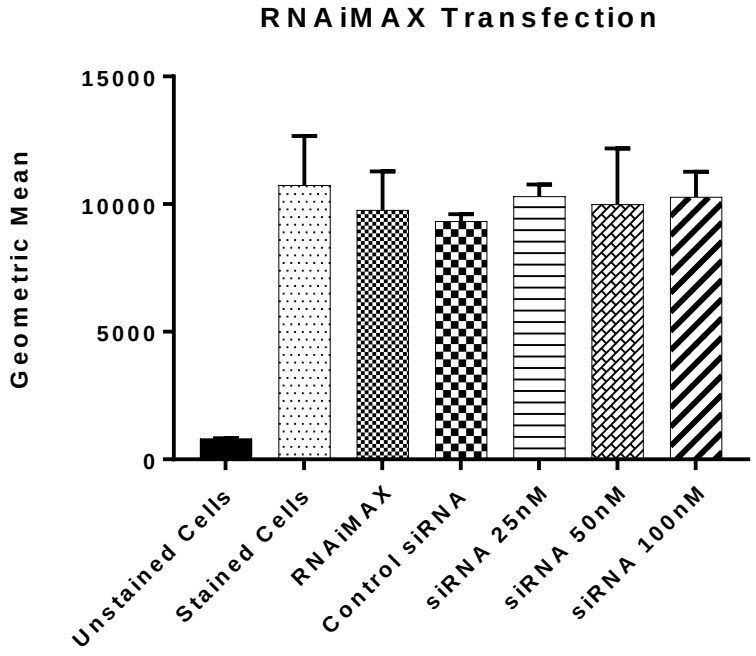

Figure 4.9 Quantitative CCR5 expression by Ghost Hi-5 cells 72 hours post transfection with RNAiMAX. Results are presented as mean ± SD (n=3).

As it is clear, and unlike what was previously apparent for the same transfection agent and siRNA, none of the tested concentrations decreased the level of CCR5 expression. Moreover, there were no significant differences between any of the tested conditions.

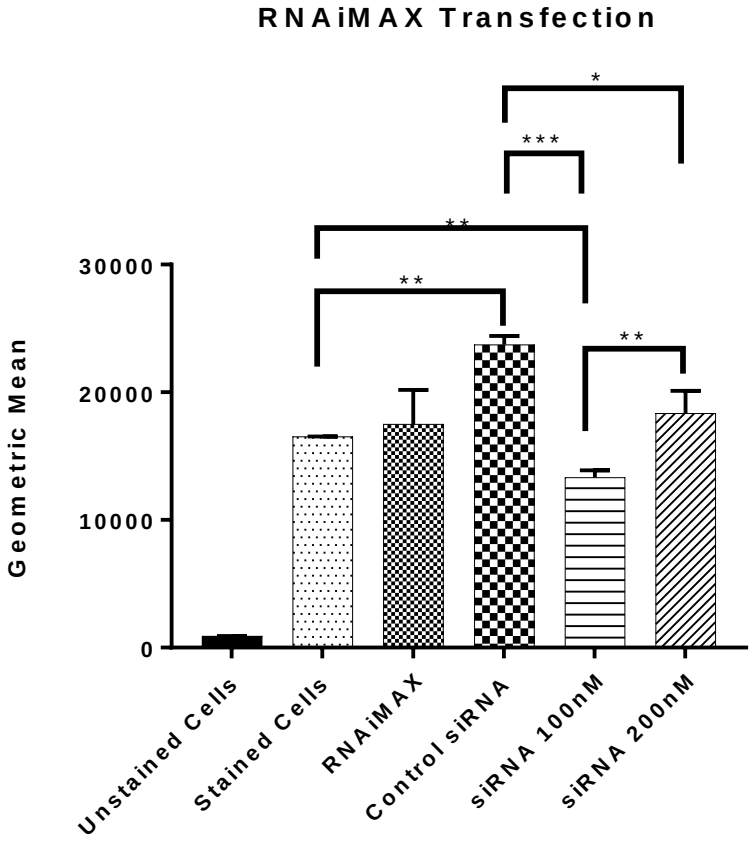

Figure 4.10 Quantitative CCR5 expression by Ghost Hi-5 cells 72 hours post transfection with RNAiMAX. Results are presented as mean ± SD (n=3). (*), (**) and (***) denote a significant difference at p<0.05, p<0.01 and p<0.005, respectively.

Interestingly, the control siRNA, but not the transfection agent alone, increased the fluorescence measured when compared to untreated cells, which did not happen in the previous experiments. Moreover, it is important to notice that, for all the three controls, fluorescence values obtained were substantially higher in the latter experiment. This lack of consistency might be due to several factors. Firstly, the Ghost Hi-5 cells used for each of these transfections were at a different passage. Even though they were within the limit for CCR5 over-expression [142], this discrepancy might cause some changes in the receptor levels at the surface. However, a more plausible explanation might be linked to the determination of the target population previously described. The used approach, sequential gating, while having the upsides of being intuitive and simple, is also very susceptible to subjectivity [143], which could help to explain these differences. More importantly, siRNA at 100 nM did cause a significant reduction in CCR5 levels when compared to untreated cells, siRNA at 200 nM and the control siRNA. However, no statistical significance was attained when comparing the effect of RNAiMAX

alone and siRNA at 100 nM, which does not allow to certainly conclude regarding the ability of this siRNA to silence CCR5 expression. The fact that cells treated with 200 nM express significantly more CCR5 than cells treated with 100 nM seems to indicate that the effective ability of this siRNA to silence this receptor, even if not as strong as expected and desired, is higher for 100 nM than for 200 nM.

All these inconsistencies regarding the experiments with this transfection agent could indicate that the siRNA being used was not as potent as desired, or that it was not in proper conditions to effectively silence the expression of CCR5.

4.1.5 siRNA (TFS)

In order to assess whether the siRNA (SCB) used up until this point was indeed faulty, a different commercial siRNA, with a distinct nucleotide sequence, from now on referred to as (TFS), was tested with RNAiMAX, since it was the transfection reagent that apparently yielded the most promising results. Obtained fluorescence values are shown in **Figure 4.11**.

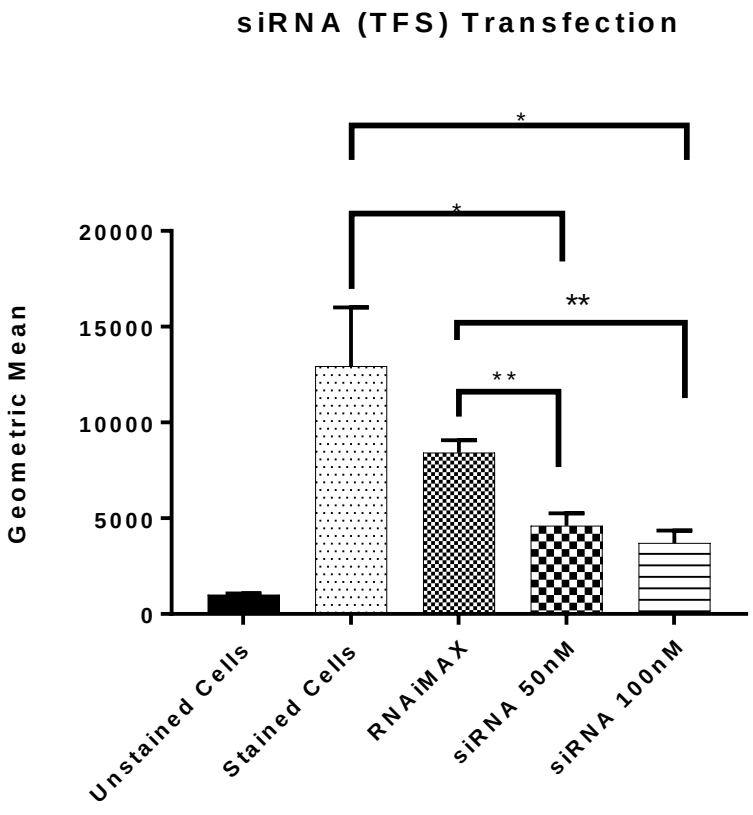

Figure 4.11 Quantitative CCR5 expression by Ghost Hi-5 cells 72 hours post transfection with RNAiMAX. Results are presented as mean ± SD (n=3). (*) and (**) denote a significant difference at p<0.05 and p<0.01, respectively.

Both 50 and 100 nM treatments lead to a significant decrease in the fluorescence signal measured, when compared to both untreated cells and cells incubated with RNAiMAX alone. Moreover, there were no significant differences between the two siRNA concentrations tested or between the two control groups (stained cells and cells treated with RNAiMAX alone). Taking into account that this was a first experiment, and that there was no optimization process, the results obtained seem to clearly indicate that this new siRNA, unlike the previous one, silenced the expression of CCR5 in a relatively potent fashion. Further optimizations, particularly regarding incubation time and siRNA concentrations, as well as the possibility of performing a second transfection 24 hours after the initial one, could lead to an even more pronounced silencing of CCR5 expression.

Since the ultimate goal of this work was to incorporate a siRNA able to silence CCR5 in NPs, and study the formulation ability to deliver the siRNA to Ghost Hi-5 cells, and consequently decrease the expression of the receptor, siRNA (TFS) was chosen to be used in the following experiments.

4.2 Production and characterization of NPs

PLGA-PEG-based NPs with different internal aqueous phases (see Chapter 3) were produced by double emulsion solvent evaporation technique. This technique is commonly used for obtaining polymeric NPs for drug delivery application, particularly when incorporation of hydrophilic compounds is desired [144, 145]. In order to achieve higher association of siRNA to the NPs, the siRNA was pre-complexed with polyamines prior to the NPs production, as previously described by Woodrow *et al*. [6]. Data on the characterization of produced NPs are presented in the following.

4.2.1 Size and surface charge

All prepared NP formulations were analyzed by DLS, for the determination of their size (mean hydrodynamic diameter) and polydispersity index (PdI), and by ELS, for the determination of their surface charge (zeta potential). DLS measures particle size through determination of the diffusion coefficient. The Brownian motion of the particles is measured, which, through well established relationships between diffusion speed and size, allows the determination of the hydrodynamic diameter of the particles. On the other hand, when using ELS, the velocity of a charged particle under an applied electric field is used to measure the

charge of the diffuse layer around the particle. This feature is expressed as the zeta potential, which provides an indirect measure of the surface charge [146].

4.2.1.1 Surfactant

PLGA-PEG was selected to be tested for the incorporation of the siRNA, being a copolymer that has been extensively reported as a good carrier for nucleic acids, among other active molecules, due to its good biodegradability and biocompatibility [147-150]. In order to decide whether or not to use poloxamer 407 as a surfactant, blank PLGA-PEG NPs (i.e., without the incorporation of siRNA-polyamine complexes), both with and without the referred surfactant, at a concentration of 0.2% (w/v), were produced and characterized in terms of size and surface charge. Data obtained are shown in **Table 4.1**.

Table 4.1 Size, polydispersity index and zeta potential values for NP formulations (n=1; SD corresponds to the three measurements performed by DLS for a single sample).

Formulation	Hydrodynamic diameter (nm)	PdI	Zeta potential (mV)
NPs w/o surfactant	203.7 ± 1.3	0.187 ± 0.024	-4.8 ± 0.4
NPs w/ surfactant	138.0 ± 0.4	0.110 ± 0.009	-4.9 ± 0.5

An increase in terms of average diameter was observed, while PdI values were higher in NPs produced without stabilizer. Surface charge was virtually the same, indicating that NPs obtained were nearly neutral. Zeta potential values ranging from -5mV to 5mV are broadly considered to be a consequence of dense PEGylation [151].

It has been reported that, in order to overcome mucosal barriers, designing NPs with size similar to that of virus that are able to efficiently overcome those same obstacles represents an attractive approach [152-154]. Thus, NPs with a hydrodynamic size below 200 nm are desired for intravaginal delivery. Even though the NPs without surfactant present an acceptable size in that perspective, the formulation containing poloxamer 407 was selected for further experiments, particularly due to the relatively lower PdI obtained.

4.2.1.2 Low siRNA (SCB) concentration

In parallel with the first siRNA transfections, NPs containing siRNA (SCB) were initially produced. Besides NPs with siRNA alone or complexed with either putrescine or spermidine at

a N/P ratio of 8/1, blank NPs were obtained as a control. For these batches, the amount of siRNA per formulation was 200 pmol. Results obtained are presented in **Table 4.2**.

Table 4.2 Size, polydispersity index and zeta potential values for NP formulations (n=1; SD corresponds to the three measurements performed by DLS for a single sample).

Formulation	Hydrodynamic diameter (nm)	PdI	Zeta potential (mV)
Blank NPs	107.6 ± 0.4	0.162 ± 0.010	-4.9 ± 0.3
l-siRNA NPs	116.3 ± 4.7	0.225 ± 0.004	-4.3 ± 0.7
Putrescine 8/1 NPs	95.6 ± 1.1	0.277 ± 0.020	-7.4 ± 1.7
l-Spermidine 8/1 NPs	124.9 ± 2.0	0.119 ± 0.018	-4.4 ± 0.3

Overall, the NPs obtained were similar regarding the three considered parameters. One of the major differences observed is the fact that NPs loaded with siRNA pre-complexed with putrescine appear to be slightly smaller and more negative. However, these differences are likely to be of reduced importance, as they are unlikely to have an impact biologically. Particles loaded with siRNA pre-complexed with spermidine presented the lowest PdI, even when compared with blank NPs. On the other hand, the value obtained for this parameter for both siRNA-putrescine and siRNA-only loaded formulations seem to indicate populations with wider dispersion in terms of size.

4.2.1.3 High siRNA (TFS) concentration

In posterior NPs productions, a higher quantity of siRNA (TFS) (1 nmol) was associated with the NPs. Furthermore, besides the N/P ratio of 8/1, a lower ratio, 3/1, that has also been described in literature to significantly improve siRNA association with polymeric NPs [6], was tested as well. Data obtained for these formulations is synthetized in **Table 4.3**.

Table 4.3 Size, polydispersity index and zeta potential values for NP formulations.

Formulation	Hydrodynamic diameter (nm)	PdI	Zeta potential (mV)	n
Blank NPs	135.0 ± 11.8	0.117 ± 0.022	-5.7 ± 1.2	5
h-siRNA NPs	136.1 ± 7.2	0.110 ± 0.020	-6.5 ± 0.6	5
h-Spermidine 8/1 NPs	146.2 ± 16.2	0.105 ± 0.027	-5.9 ± 1.2	5
Spermidine 3/1 NPs	157.7 ± 3.2	0.099 ± 0.015	-6.5 ± 0.6	3

Overall, data seemed to be consistent with what was obtained for previous formulations. The slight increase observed in the particles size could be indicative of successful siRNA incorporation. However, the fact that blank NPs also appear to be larger make this assertation impossible to be concluded. A modest decrease regarding zeta potential of siRNA loaded NPs, when compared with the previously obtained formulations, can be observed. While this could be indicative of a higher amount of siRNA associated, which is very likely, since the amount of siRNA per formulation was five times higher than in the previous experiment, such a slight change is unlikely to be an important factor. Moreover, a similar decrease was observed in blank NPs, which might suggest that the obtained differences are somewhat negligible. Moreover, the different N/P ratios also did not seem to alter the colloidal properties of the particles. In sum, this seems to indicate that the general formulation is robust.

4.2.2 siRNA content

In order to assess the efficacy of the siRNA incorporation, lyophilized NPs were destroyed and the aqueous fraction, containing the siRNA, was extracted and analyzed regarding siRNA content, following a protocol adapted from Woodrow *et al*. [6]. In order to quantify the amount of siRNA extracted, SYBR Gold, a probe that binds to the phosphate backbone of the nucleic acid and has been used to quantify siRNA in NPs formulations, was incubated with the referred aqueous phase, and fluorescence at specific wavelengths was measured.

To establish a correlation between the fluorescence and siRNA concentrations, two sets of standard curves, with known concentrations of siRNA, were obtained. The first set was obtained measuring the fluorescence of siRNA alone, while the second was attained measuring the fluorescence of siRNA in the presence of blank NPs extract. Moreover, the fluorimeter used for the measurements allowed two different methods for fluorescence measuring: EndPoint and

AreaScan. While the first approach results in the output of only the last spot read for each well, the latter reads several spots and provides the average value.

4.2.2.1 Low siRNA (SCB) concentration

Despite the fact that siRNA (SCB) was not effectively silencing the expression of CCR5, it was hypothesized that it could still be detected through fluorescence, since the probe used is generic and binds to the phosphate backbone. This, theoretically, could allow the siRNA to be detected, even if not functional. Since the amount of siRNA incorporated was 200 pmol per formulation, and taking into account the extraction process, the maximum siRNA concentration per sample was 40 nM. Standard curves were obtained based on fluorescence values depicted in **Table 4.4**.

Table 4.4 Fluorescence values, obtained for each acquisition procedure and control, used to obtain standard curves.

siRNA (nM) / Control	0	2.5	5	10	20	50	100
EndPoint siRNA	14	13	11	25	29	62	157
EndPoint siRNA+NPs	16	8	15	29	38	101	181
AreaScan siRNA	7	12	12	22	31	62	147
AreaScan siRNA+NPs	14	20	20	30	48	102	176

Despite SYBR Gold being regarded as extremely sensitive, to the point of being able to quantify siRNA at 0.5 nM, according to manufacturer indications, it seems clear from the values of fluorescence obtained for the lower siRNA concentrations that this method of quantification is not reliable for concentrations below 10 nM. Furthermore, particularly regarding EndPoint acquisition, fluorescence from 10 and 20 nM are almost indistinguishable. It seems clear, then, that higher concentrations of siRNA must be used in order to attain more reliable standard curves. From the above values, the standard curves presented in **Figure 4.12** were obtained.

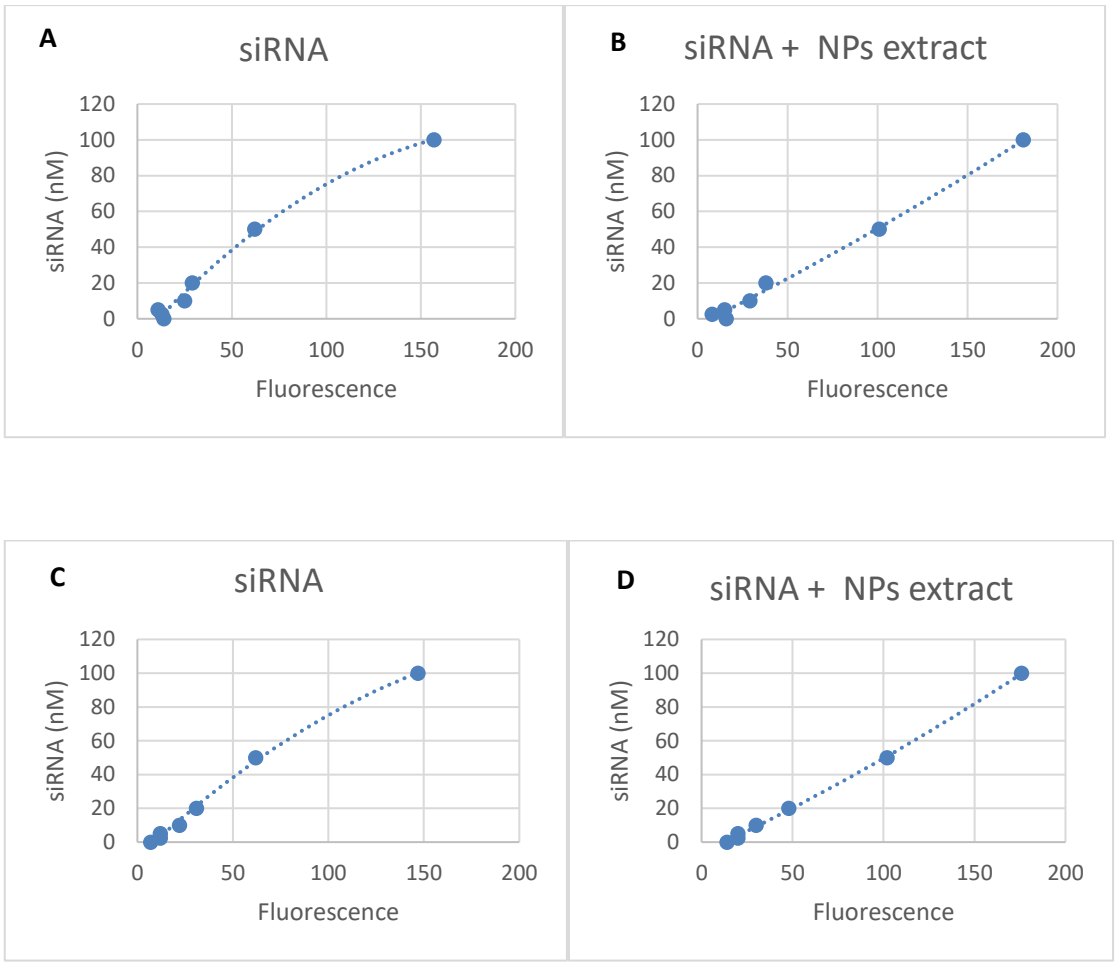

Figure 4.12 Standard curves obtained correlating fluorescence measured and siRNA concentrations. (A) Standard curve obtained through EndPoint readings of siRNA alone. (B) Standard curve obtained through EndPoint readings of siRNA with NPs extract. (C) Standard curve obtained through AreaScan readings of siRNA alone. (D) Standard curve obtained through AreaScan readings of siRNA with NPs extract.

The equations obtained from each standard curve, which were applied in order to correlate fluorescence values and siRNA concentration, as well as the corresponding values of R^2, were as follows:

$y = -0.0028 * x^2 + 1.1563 * x - 12.418$ $R^2 = 0.9922$ (Eq. 1)

$y = 0.0004 * x^2 + 0.5001 * x - 3.482$ $R^2 = 0.9945$ (Eq. 2)

$y = -0.0021 * x^2 + 1.05 * x - 9.0478$ $R^2 = 0.9972$ (Eq. 3)

$y = 0.0006 * x^2 + 0.5034 * x - 6.3966$ $R^2 = 0.9993$ (Eq. 4)

Equations (1), (2), (3) and (4) were obtained from the standard curves depicted in **Figure 4.12** (A), (B), (C) and (D), respectively.

For each formulation using siRNA (SCB), ten replicates were evaluated for their siRNA content. The values obtained are presented in **Table 4.5**.

Table 4.5 Fluorescence values obtained for each formulation with EndPoint and AreaScan. Results are presented as mean ± SD (n=10).

Formulation	EndPoint	AreaScan
Blank NPs	15.0 ± 4.7	12.1 ± 2.7
l-siRNA NPs	13.7 ± 4.3	15.1 ± 2.7
Putrescine 8/1 NPs	12.0 ± 3.1	12.3 ± 1.1
l-Spermidine 8/1 NPs	18.1 ± 3.3	19.1 ± 3.6

Using the mathematic expressions obtained from the standard curves, these fluorescence values were used to calculate the siRNA concentration present in each well. Furthermore, association efficiencies were also calculated based on these data. The obtained values are presented in **Tables 4.6** and **4.7**.

Table 4.6 siRNA concentrations (nM) calculated based on obtained fluorescence values. Results are presented as mean ± SD (n=10).

Formulation	EndPoint siRNA	EndPoint siRNA+NPs	AreaScan siRNA	AreaScan siRNA+NPs
Blank NPs	4.2 ± 5.0	4.1 ± 2.4	3.3 ± 2.7	-0.2 ± 1.4
l-siRNA NPs	2.9 ± 4.7	3.5 ± 2.2	6.3 ± 2.6	1.4 ± 1.4
Putrescine 8/1 NPs	1.0 ± 3.4	2.6 ± 1.6	3.6 ± 1.1	-0.1 ± 0.6
l-Spermidine 8/1 NPs	7.6 ± 3.5	5.7 ± 1.7	10.2 ± 3.4	3.4 ± 1.9

Table 4.7 Association efficiencies (%) calculated based on obtained siRNA concentrations. Results are presented as mean ± SD (n=10).

Formulation	EndPoint siRNA	EndPoint siRNA+NPs	AreaScan siRNA	AreaScan siRNA+NPs
Blank NPs	10.6 ± 12.6	10.3 ± 6.0	8.3 ± 6.6	-
l-siRNA NPs	7.1 ± 11.7	8.6 ± 5.5	15.8 ± 6.6	3.4 ± 3.5
Putrescine 8/1 NPs	2.6 ± 8.6	6.5 ± 4.0	8.9 ± 2.7	-
l-Spermidine 8/1 NPs	18.9 ± 8.6	14.3 ± 4.2	25.5 ± 8.6	8.6 ± 4.7

Out of the two reading approaches, AreaScan appears to be more reliable than EndPoint, given the less variability of values obtained for the fluorescence. Moreover, the second standard curve used, which was obtained adding siRNA with extract of blank NPs, also appears to provide more consistent results than using siRNA by itself to establish the standard curve.

From the standard curves, it further seems clear that siRNA concentrations under 10 nM are not quantifiable or even detectable using this technique. Moreover, since the values of fluorescence obtained from the samples are very close or, in some cases, even lower than the background levels, not much can be said regarding these results. However, it appears that values obtained for NPs loaded with siRNA-spermidine, even if low and close to background levels, are consistently higher than those obtained for siRNA-putrescine complexes. In fact, blank and siRNA-putrescine loaded NPs present very similar results. This is also in agreement with the report of Woodrow *et al*. [6]. Therefore, for further experiments, only spermidine was used for producing siRNA-loaded NPs. Regarding association efficiencies, very low values were obtained. Using the more reliable standard curve attained, association efficiency for NPs loaded with siRNA complexed with spermidine presented the higher efficiency, only 8.6%. In order to obtain higher association, as well as to obtain values considerably higher than the background, 1nmol, rather than 200pmol, of siRNA were used per formulation in further experiments.

4.2.2.2 High siRNA (TFS) concentration

When using siRNA (TFS), 1 nmol per formulation was associated with the NPs. Taking into account the extraction and detection processes, the maximum siRNA concentration per sample was 200 nM. The same rationale to correlate the measured fluorescence and siRNA content was used. However, in order to obtain the standard curves, siRNA at 5 nM, rather than 2.5 nM, was the lowest concentration measured. Moreover, since for these experiments the maximum

obtainable siRNA content was 200 nM, the fluorescence value for this concentration was also assessed and used in the attainment of standard curves.

Firstly, three different formulations were obtained: blank NPs, h-siRNA NPs and h-Spermidine 8/1 NPs. Similarly to what was performed in the previous siRNA quantification, standard curves were obtained based on fluorescence values obtained for known siRNA concentration. These are depicted in **Table 4.8.**

Table 4.8 Fluorescence values, obtained for each acquisition procedure and control, used to obtain standard curves.

siRNA (nM) / Control	0	5	10	20	50	100	200
EndPoint siRNA	6	46	86	168	352	458	659
EndPoint siRNA+NPs	53	146	237	203	385	531	705
AreaScan siRNA	4	47	86	148	292	419	648
AreaScan siRNA+NPs	58	99	146	216	431	526	699

Similarly to what had happened in the previous siRNA quantification, the AreaScan approach provided more consistent results than EndPoint. For instance, with EndPoint, the fluorescence value obtained for siRNA at 20 nM was, for one of the standard curves, lower when compared to siRNA at 10 nM. Fluorescence values measured for each formulation are shown in **Table 4.9**.

Table 4.9 Fluorescence values obtained for each formulation with EndPoint and AreaScan. Results are presented as mean ± SD (n=10).

Formulation	EndPoint	AreaScan
Blank NPs	37.8 ± 31.5	36.6 ± 13.0
h-siRNA NPs	49.9 ± 18.0	52.0 ± 20.0
h-Spermidine 8/1 NPs	13.4 ± 3.4	12.9 ± 3.0

Since the values obtained for siRNA-spermidine complexes were incredibly low, even lower than the background, the aqueous phase that was extracted was submitted to an extra round of vortexing and centrifugation. Following the same procedure for siRNA quantification, the results obtained were as follows in **Table 4.10**.

Table 4.10 Fluorescence values obtained for each formulation with EndPoint and AreaScan. Formulation marked with "*" refer to samples submitted to two rounds of vortexing and centrifugation during the extraction procedure. Results are presented as mean ± SD (n=10).

Formulation	EndPoint	AreaScan
h-Spermidine 8/1 NPs*	133.6 ± 52.3	189.4 ± 65.5

Mathematic expressions obtained from the standard curves were used to calculate the siRNA concentration present in each well. The obtained values are presented in **Table 4.11**.

Table 4.11 siRNA concentrations (nM) calculated based on obtained fluorescence values. Formulation marked with "*" refer to samples submitted to two rounds of vortexing and centrifugation during the extraction procedure. Results are presented as mean ± SD (n=10).

Formulation	EndPoint siRNA	EndPoint siRNA+NPs	AreaScan siRNA	AreaScan siRNA+NPs
Blank NPs	5.0 ± 1.4	4.0 ± 0.8	3.5 ± 1.5	9.0 ± 1.2
h-siRNA NPs	5.0 ± 1.0	3.3 ± 0.3	5.4 ± 2.7	8.2 ± 1.2
h-Spermidine 8/1 NPs	4.2 ± 0.0	4.9 ± 0.2	1.0 ± 0.3	11.7 ± 0.4
h-Spermidine 8/1 NPs*	12.8 ± 7.3	5.6 ± 4.0	31.7 ± 16.3	11.4 ± 7.4

Comparing the siRNA concentrations obtained with both AreaScan standard curves, it is noted that the standard curve obtained with siRNA and extract of blank particles was not reliable. In fact, when using the mathematic expression obtained from this curve, the concentration obtained for the mean fluorescence of spermidine 8/1 NPs, 12.9, was 11.57 nM. On the other hand, for siRNA NPs, which presented higher fluorescence, more precisely 52.0, the concentration of siRNA obtained was only 8.2 nM. Based on this, the standard curve obtained through AreaScan with siRNA only appeared to be the most reliable in correlating fluorescence values with siRNA concentration, and, ultimately, association efficiencies, which are presented in **Table 4.12**.

Table 4.12 Association efficiencies (%) calculated based on obtained siRNA concentrations. Formulation marked with "*" refer to samples submitted to two rounds of vortexing and centrifugation during the extraction procedure. Results are presented as mean ± SD (n=10).

Formulation	EndPoint siRNA	EndPoint siRNA+NPs	AreaScan siRNA	AreaScan siRNA+NPs
Blank NPs	2.5 ± 0.7	2.0 ± 0.4	1.7 ± 0.7	4.5 ± 0.6
h-siRNA NPs	2.5 ± 0.5	1.6 ± 0.1	2.7 ± 1.4	4.1 ± 0.6
h-Spermidine 8/1 NPs	2.1 ± 0.0	2.4 ± 0.1	0.5 ± 0.1	5.8 ± 0.2
h-Spermidine 8/1 NPs*	6.7 ± 3.7	2.8 ± 2.0	15.8 ± 8.2	5.7 ± 3.7

While the fluorescence values, and corresponding siRNA concentrations, were still lower than pretended, the difference to what was obtained prior to the second round of vortexing and centrifugation was substantial. This appears to indicate that the extraction procedure used until this point was not robust enough. Thus, in the following quantification experiments, two rounds of vortexing and centrifugation, rather than one, were performed.

Given the fact that, unlike siRNA-spermidine loaded NPs, siRNA NPs and blank NPs were not submitted to the extra vortexing and centrifugation, it is not correct to interpreter the obtained data as reliable. However, regarding the measured fluorescence values obtained from the standard curves, and comparing with those obtained in the previous experiment, with siRNA (SCB), it seems safe to conclude that this siRNA, on top of being extremely faulty in terms of function, was also not quantifiable by this method, as was firstly hypothesized.

In order to better understand the effect of an extra round of vortexing and centrifugation, both cases, either one or two rounds, were tested. Besides this modification in the extraction procedure, the attainment of standard curves was also altered. Instead of using either siRNA by itself or siRNA with extract of blank particles, siRNA-spermidine complexes and siRNA-spermidine complexes with extract of blank NPs were used. In **Table 4.13** fluorescence values obtained to obtain standard curves are presented.

Table 4.13 Fluorescence values, obtained for each acquisition procedure and control, used to obtain standard curves.

siRNA (nM) / Control	0	5	10	20	50	100	200
EndPoint siRNA-Spermidine	12	63	108	184	338	589	1058
EndPoint siRNA-Spermidine+NPs	22	93	142	256	486	681	836
AreaScan siRNA-Spermidine	7	57	105	192	317	543	1055
AreaScan siRNA-Spermidine+NPs	24	86	135	263	501	710	864

As was the case with the previous quantification, the standard curve obtained with NPs extract was not trustworthy. For instance, when using the mathematic expression obtained from this curve, the concentration obtained for blank NPs was 6.8 nM, whether submitted to one or two rounds of vortexing and centrifugation (**Table 4.15**). However, as can be seen in **Table 4.14**, the fluorescence obtained for each cases were not similar (61.6 and 141.2). Based on this, the other standard curve, obtained by AreaScan with siRNA-spermidine only, was taken as more reliable. In this experiment, five replicates were performed for each condition. The fluorescence values obtained are shown in **Table 4.14**.

Table 4.14 Fluorescence values obtained for each formulation with EndPoint and AreaScan. Formulations marked with "*" refer to samples submitted to two rounds of vortexing and centrifugation during the extraction procedure. Results are presented as mean ± SD (n=5).

Formulation	EndPoint	AreaScan
Blank NPs	72.0 ± 25.7	61.6 ± 24.0
h-siRNA NPs	110.0 ± 23.4	122.2 ± 28.1
h-Spermidine 8/1 NPs	123.6 ± 79.6	117.4 ± 76.8
Blank NPs*	250.6 ± 195.6	141.2 ± 63.7
h-siRNA NPs*	424 ± 163.5	409.4 ± 117.5
h-Spermidine 8/1 NPs*	427.2 ± 168.9	305.2 ± 107.7

Similar to what was observed in the previous experiment, there was a significant increase in the fluorescence values obtained, both for NPs loaded with siRNA alone and siRNA complexed with spermidine, submitted to two rounds of vortexing and centrifugation rather than one. This resulted in a 3-fold increase in terms of siRNA concentration. Interestingly, a similar increase was observed in blank NPs.

Using the mathematic expressions obtained from the standard curves, the siRNA concentrations present in each well were calculated. The obtained values are presented in **Table 4.15**.

Table 4.15 siRNA concentrations (nM) calculated based on obtained fluorescence values. Formulations marked with "*" refer to samples submitted to two rounds of vortexing and centrifugation during the extraction procedure. Results are presented as mean ± SD (n=5).

Formulation	EndPoint siRNA	EndPoint siRNA+NPs	AreaScan siRNA	AreaScan siRNA+NPs
Blank NPs	6.0 ± 4.4	6.0 ± 1.2	4.5 ± 4.2	6.8 ± 1.2
h-siRNA NPs	11.9 ± 4.1	4.9 ± 0.4	15.2 ± 5.0	5.3 ± 0.2
h-Spermidine 8/1 NPs	14.2 ± 14.3	7.1 ± 2.7	14.5 ± 13.8	7.4 ± 3.1
Blank NPs*	36.2 ± 38.7	26.2 ± 38.6	18.7 ± 11.4	6.8 ± 2.3
h-siRNA NPs*	66.0 ± 34.0	51.1 ± 47.2	68.1 ± 22.2	43.4 ± 26.1
h-Spermidine 8/1 NPs*	66.6 ± 34.6	52.6 ± 44.0	48.6 ± 19.9	22.9 ± 15.0

Association efficiencies were also calculated, based on the same values. Results are shown in **Table 4.16**.

Table 4.16 Association efficiencies (%) calculated based on obtained siRNA concentrations. Formulations marked with "*" refer to samples submitted to two rounds of vortexing and centrifugation during the extraction procedure. Results are presented as mean ± SD (n=5).

Formulation	EndPoint siRNA	EndPoint siRNA+NPs	AreaScan siRNA	AreaScan siRNA+NPs
Blank NPs	3.0 ± 2.2	3.0 ± 0.6	2.3 ± 2.1	3.4 ± 0.6
h-siRNA NPs	5.9 ± 2.0	2.4 ± 0.2	7.6 ± 2.5	2.7 ± 0.1
h-Spermidine 8/1 NPs	7.1 ± 7.1	3.6 ± 1.3	7.2 ± 6.9	3.7 ± 1.6
Blank NPs*	18.1 ± 19.3	13.1 ± 19.3	9.3 ± 5.7	3.4 ± 1.1
h-siRNA NPs*	33.0 ± 17.0	25.5 ± 23.6	34.1 ± 11.1	21.7 ± 13.1
h-Spermidine 8/1 NPs*	33.3 ± 17.3	26.3 ± 22.0	24.3 ± 9.9	11.5 ± 7.5

This experiment lead to the assumption that submitting the aqueous phase to two rounds of vortexing and centrifugation does indeed improve the efficacy of the extraction procedure. However, a similar increase in fluorescence values was observed in blank NPs. Even though these values present considerable fluctuation, while fluorescence values for replicates of both

siRNA and siRNA-spermidine samples are more consistent, it still poses as a drawback for this extraction strategy. Regarding the fluorescence values obtained, and corresponding siRNA concentrations and association efficiencies, spermidine, unlike expected, did not appear to increase the amount of siRNA associated with the NPs.

Finally, a N/P ratio of 3/1 for spermidine was also tested. In this experiment, unlike in the previous, only a fraction of the lyophilized NPs was dissolved. Two standard curves were obtained, both with siRNA-spermidine complexes and extract of blank NPs. The difference between the two curves was the N/P ratio: one curve was obtained with complexes with N/P ratio of 3/1 and other with a ratio of 8/1. Fluorescence values obtained to establish each of these standard curves are shown in **Table 4.17**.

Table 4.17 Fluorescence values, obtained for each acquisition procedure and control, used to obtain standard curves.

siRNA (nM) / Control	0	5	10	20	50	100	200
EndPoint siRNA-Spermidine 3/1	106	133	130	202	384	698	1082
EndPoint siRNA-Spermidine 8/1	52	88	110	176	326	471	653
AreaScan siRNA-Spermidine 3/1	163	160	144	246	393	716	1279
AreaScan siRNA-Spermidine 8/1	76	104	125	198	399	668	883

Since the fluorescence value for siRNA at 10 nM was lower than what was obtained for siRNA at 5 nM and for no siRNA, the standard curve with a N/P ratio of 3/1 obtained through AreaScan was unreliable. Thus, the other standard curve, obtained through the same approach, was assumed to be the more trustworthy to use in correlating fluorescence with siRNA concentrations. For each of the four formulations, three batches of NPs were produced (A, B and C), and the aqueous phase extracted from each batch was analyzed three times. Fluorescence values are shown in **Table 4.18**.

Table 4.18 Fluorescence values obtained for each formulation with EndPoint and AreaScan. Results are presented as mean ± SD (n=3).

Formulation	EndPoint	AreaScan
Blank NPs A	10.3 ± 3.9	18.0 ± 7.8
Blank NPs B	136.3 ± 14.5	158.7 ± 33.5
Blank NPs C	11.0 ± 2.2	10.3 ± 2.6
h-siRNA NPs A	141.3 ± 60.2	172.3 ± 82.3
h-siRNA NPs B	42.7 ± 13.4	37.7 ± 9.7
h-siRNA NPs C	103.3 ± 27.5	110.7 ± 33.6
Spermidine 3/1 NPs A	42 ± 11.3	50.3 ± 11.8
Spermidine 3/1 NPs B	97.7 ± 56.0	103.3 ± 55.5
Spermidine 3/1 NPs C	37.3 ± 14.4	34.7 ± 11.6
h-Spermidine 8/1 NPs A	22.3 ± 9.7	24.7 ± 6.6
h-Spermidine 8/1 NPs B	270.7 ± 113.5	224.0 ± 90.7
h-Spermidine 8/1 NPs C	147.7 ± 61.8	167.3 ± 80.9

Using the mathematic expressions obtained from the standard curves, both siRNA concentrations and association efficiencies were calculated. The obtained values are presented in **Tables 4.19** and **4.20**.

Table 4.19 siRNA concentrations (nM) calculated based on obtained fluorescence values. Results are presented as mean ± SD (n=9).

Formulation	EndPoint 3/1	EndPoint 8/1	AreaScan 3/1	AreaScan 8/1
Blank NPs	-2.2 ± 7.3	2.3 ± 0.7	-10.8 ± 12.3	6.2 ± 2.6
h-siRNA NPs	3.1 ± 7.3	2.4 ± 3.8	-3.1 ± 13.0	7.8 ± 4.8
Spermidine 3/1 NPs	-1.5 ± 5.3	1.3 ± 0.9	-10.8 ± 7.7	5.6 ± 1.5
h-Spermidine 8/1 NPs	10.5 ± 17.5	11.5 ± 18.1	2.4 ± 18.9	10.6 ± 7.7

Table 4.20 Association efficiencies (%) calculated based on obtained siRNA concentrations. Results are presented as mean ± SD (n=3).

Formulation	EndPoint 3/1	EndPoint 8/1	AreaScan 3/1	AreaScan 8/1
Blank NPs	-	1.1 ± 0.4	-	3.1 ± 1.3
h-siRNA NPs	1.5 ± 3.6	1.2 ± 1.9	-	3.9 ± 2.4
Spermidine 3/1 NPs	-	0.7 ± 0.4	-	2.8 ± 0.7
h-Spermidine 8/1 NPs	5.3 ± 8.8	5.7 ± 9.1	1.2 ± 9.4	5.3 ± 3.8

The fact that in this experiment only a fraction of lyophilized NPs was used for the quantification assay renders the obtained data somewhat unreliable. However, a tendency can be detected in the values of fluorescence that two out of three batches (B and C) containing siRNA-spermidine at a N/P ratio of 8/1 appear to, in fact, present a considerable amount of siRNA. In spite of that, the high variability that exists in most of the values obtained, and the low trustworthiness that comes from the standard curves obtained, do not allow for definite and assertive conclusions to be retrieved from this experiment.

Overall, there was no consistency in the quantification results. Even within the same batch, very distinct values of fluorescence were obtained. One possible explanation is the fact that the mass retrieved from each batch after lyophilization was quite variable, likely due to losses during the freeze-drying process. That led to different quantities of siRNA from each formulation being quantified, which represents an additional variable that could exacerbate errors. However, even in the initial experiments, where there were no significant losses of mass during the lyophilization process, consistency was also not achieved, as can be confirmed by the variability of fluorescence values measured. Another major factor that might contribute to this lack of consistent results might be related to the process of extraction of the aqueous phase after dissolving the particles. For instance, when comparing fluorescence values

obtained from aqueous phases submitted to either one or two rounds of vortexing and centrifugation, it can be seen that not only the fluorescence of siRNA-loaded formulations increased, as it also happened for blank NPs. This might indicate that the probe used for siRNA quantification might not have high enough specificity in order to correctly quantify siRNA present in these complex formulations.

In sum, the techniques applied in this work for siRNA extraction and quantification revealed themselves to be unattractive, particularly in the range of concentrations that was used.

4.3 NPs siRNA (TFS) delivery to cultured cells

Each formulation was delivered at four different concentrations to cultured cells, in triplicate. The highest concentration used, 2.0 mg/mL, theoretically corresponds to siRNA at 100 nM. FACS data were treated just like aforementioned. Fluorescence values obtained are summarized in **Figure 4.13**.

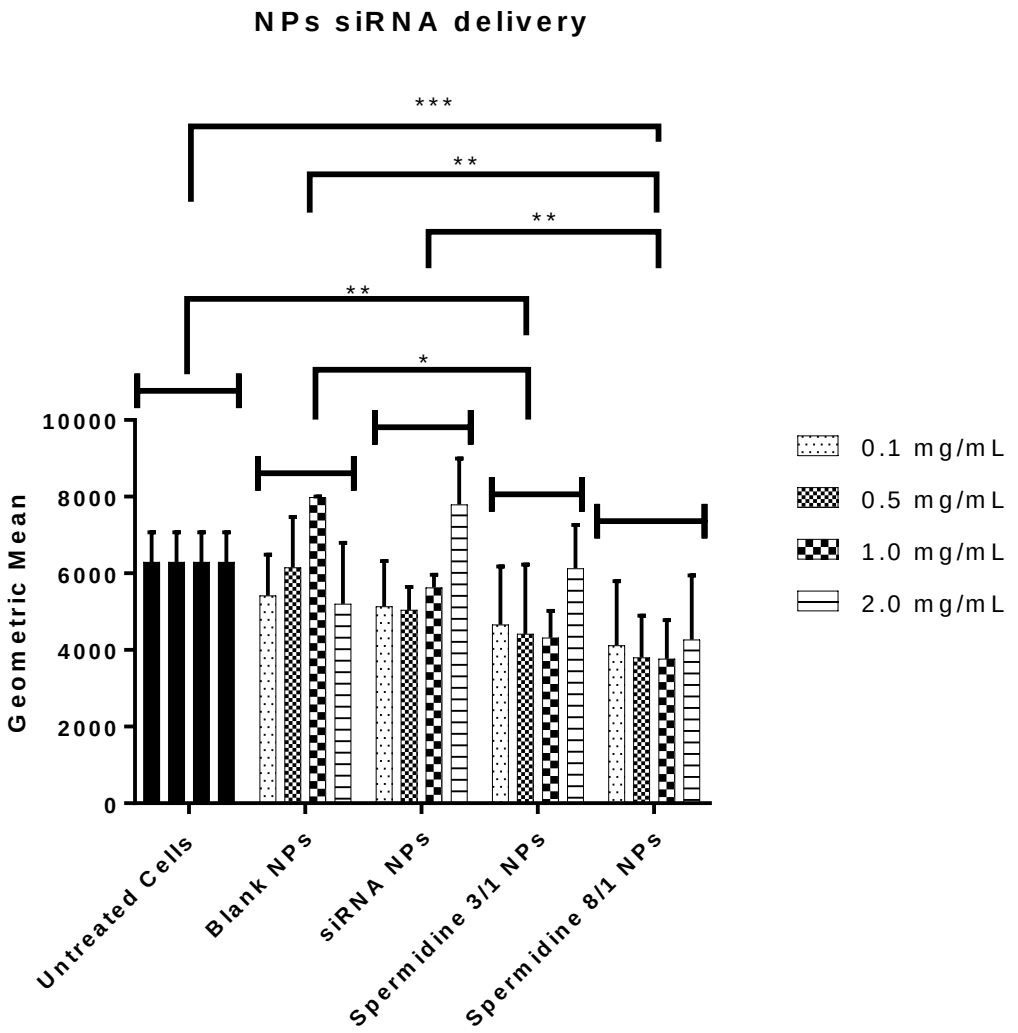

Figure 4.13 Quantitative CCR5 expression by Ghost Hi-5 cells 72 hours post incubation with NPs. In this case, one-way analysis of variance (ANOVA) was used to compare multiple groups. Results are presented as mean ± SD (n=3). (*), (**) and (***) denote a significant difference at $p<0.05$, $p<0.01$ and $p<0.005$, respectively.

Formulations containing siRNA complexed with spermidine with a N/P ratio of 8/1 significantly decreased the fluorescence measured when compared with siRNA and blank NPs, and untreated cells. On the other hand, spermidine 3/1 NPs significantly silence CCR5 expression when compared to untreated cells and blank NPs, but not siRNA NPs. These appear to not suppress CCR5 expression when compared to both cells treated with blank NPs and untreated cells. Blank NPs effect on CCR5 expression was, just like observed for the transfection agents used previously, somewhat random, since they increase the fluorescence read for cells treated with 1mg/mL, but slightly decrease when given in other concentrations. Moreover, differences in concentration seem to have little to no impact at all in the receptor expression. Interestingly, cells treated with 2 mg/mL appear to express more CCR5 than cells treated with any other concentrations, except for blank NPs.

These data might suggest that both formulations containing siRNA complexed with spermidine, particularly at a N/P ratio of 8/1, are capable of delivering siRNA to cultured Ghost Hi-5 cells, and consequently leading to mild CCR5 silencing, in similar fashion when compared with a traditional, well established and commercial transfection agent. In fact, by comparing the histograms obtained, for the same siRNA concentration, with RNAiMAX and the produced NPs, formulations with siRNA-spermidine complexes at a N/P ratio of 8/1 yielded similar silencing of CCR5, according to statistics provided by the analysis software. These data are shown in **Figures 4.14** and **4.15**.

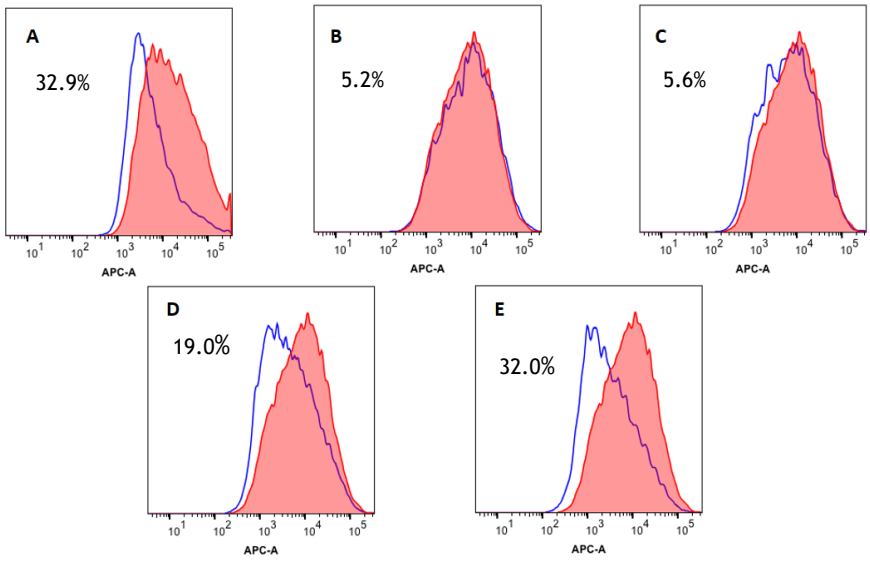

Figure 4.14 Comparison of geometric mean fluorescence between histograms of samples (blue line) and merged controls (red line, shaded). (A) CCR5 expression after transfection with commercial transfection agent; (B) CCR5 expression after incubation with Blank NPs; (C) CCR5 expression after incubation with siRNA NPs; (D) CCR5 expression after incubation with siRNA-spermidine 3/1 NPs; (E) CCR5 expression after incubation with siRNA-spermidine 8/1 NPs. Incubation time was 72h and siRNA concentration 50 nM for all formulations (1.0mg/mL for NPs formulations). Percentages shown refer to differences in the histograms provided by the analysis software, FlowJo v10.0.7.

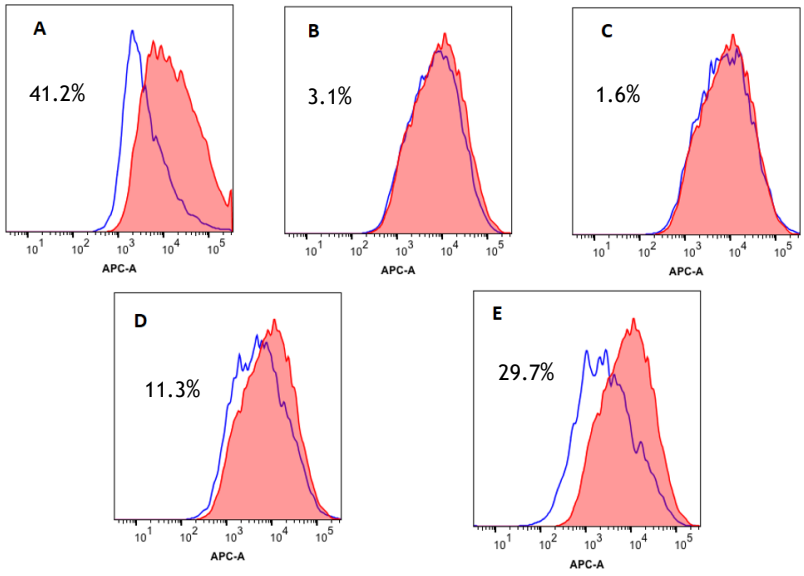

Figure 4.15 Comparison of geometric mean fluorescence between histograms of samples (blue line) and merged controls (red line, shaded). (A) CCR5 expression after transfection with commercial transfection agent; (B) CCR5 expression after incubation with Blank NPs; (C) CCR5 expression after incubation with siRNA NPs; (D) CCR5 expression after incubation with siRNA-spermidine 3/1 NPs; (E) CCR5 expression after incubation with siRNA-spermidine 8/1 NPs. Incubation time was 72h and siRNA concentration 100 nM for all formulations (2.0mg/mL for NPs formulations). Percentages shown refer to differences in the histograms provided by the analysis software, FlowJo v10.0.7.

Interestingly, siRNA concentration does not seem to alter spermidine 8/1 NPs ability to reduce CCR5 expression, since all of the four concentrations tested resulted in similar silencing. However, for the other two formulations containing siRNA, the lower concentrations (5 and 25 nM) seem to inhibit the receptor expression in stronger fashion when compared to the higher siRNA concentrations, as can be seen in **Figures 4.16** and **4.17**. Regarding blank NPs, the effect on CCR5 levels is, as expected, extremely negligible and independent of concentration.

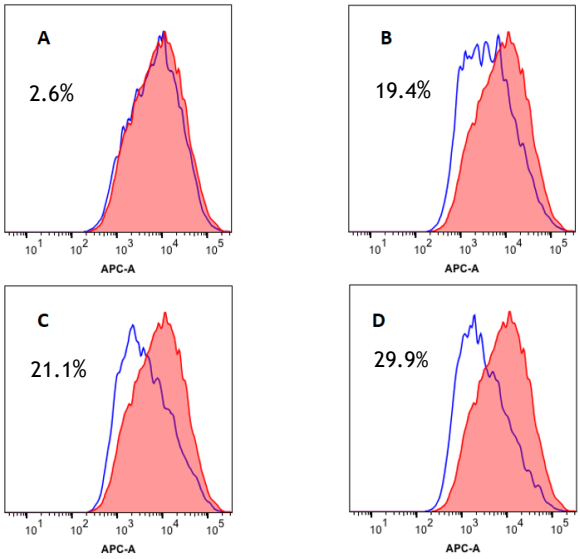

Figure 4.16 Comparison of geometric mean fluorescence between histograms of samples (blue line) and merged controls (red line, shaded). (A) CCR5 expression after incubation with Blank NPs; (B) CCR5 expression after incubation with siRNA NPs; (C) CCR5 expression after incubation with siRNA-spermidine 3/1 NPs; (D) CCR5 expression after incubation with siRNA-spermidine 8/1 NPs. Incubation time was 72h and NPs concentration 0.1mg/mL for all formulations. Percentages shown refer to differences in the histograms provided by the analysis software, FlowJo v10.0.7.

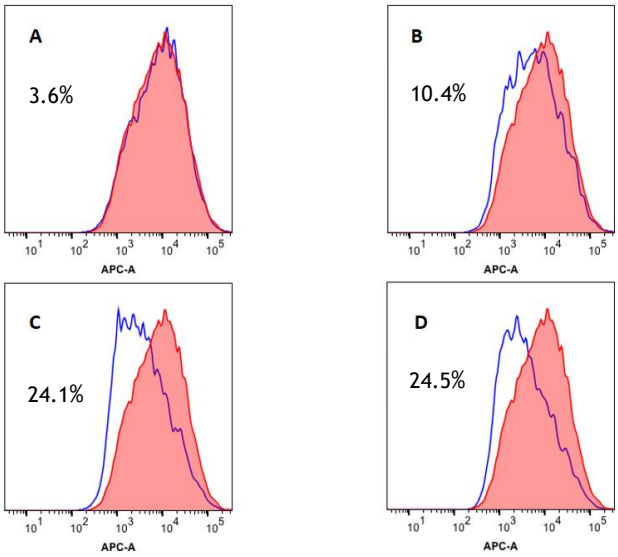

Figure 4.17 Comparison of geometric mean fluorescence between histograms of samples (blue line) and merged controls (red line, shaded). (A) CCR5 expression after incubation with Blank NPs; (B) CCR5 expression after incubation with siRNA NPs; (C) CCR5 expression after incubation with siRNA-spermidine 3/1 NPs; (D) CCR5 expression after incubation with siRNA-spermidine 8/1 NPs. Incubation time was 72h and NPs concentration 0.5mg/mL for all formulations. Percentages shown refer to differences in the histograms provided by the analysis software, FlowJo v10.0.7.

These data seem to suggest that when associated within these formulations, this siRNA is capable of silencing the same levels of CCR5 even at lower concentrations than 50 and 100 nM. In order to be able to compare these NPs ability to knockdown this receptor with complexes formed with commercial transfection agents, more studies and optimizations, regarding both carriers, need to be undertaken.

CHAPTER 5

Conclusions and Future Work

In this work, polymeric nanocarriers containing siRNA were developed. These nanoparticles, based on PLGA-PEG copolymer, were designed as a potential delivery system of siRNA with the objective of selectively silencing the expression of cell membrane receptor CCR5.

Firstly, the ability of a commercially available siRNA from SCB to silence the expression of this receptor was assessed. Three different transfection agents were used in these experiments. Although some level of silencing was attained, due to extensive optimization of the process, the expression levels of CCR5 obtained were still much higher than what can be found in literature [96]. With that in mind, a second commercial siRNA from TFS, with a different nucleotide sequence, was also tested regarding its ability to knockdown CCR5. In fact, and despite little to no optimization being done regarding this siRNA, substantially stronger CCR5 silencing was attained. Based on this, it is possible to conclude that the first siRNA tested did not have the ability to properly knockdown this receptor. Moreover, during the development process, it was verified that the monoclonal antibody used in this work to quantify the expression of CCR5 was, unlike others reported in the literature [129], indeed selective towards this receptor.

PLGA-PEG NPs containing the selected siRNA, either alone or pre-complexed with a polyamine (putrescine or spermidine), were successfully obtained through the double emulsion solvent evaporation technique. Colloidal properties of these particles seemed to be mostly unaltered despite the association of the nucleic acid, presenting similar hydrodynamic diameter and zeta potential values. Furthermore, NPs presented low PdI, and thus can be considered relatively monodisperse. Overall, these properties can be considered attractive for reaching HIV-susceptible cells at the mucosa, posterior to penetration through mucosal fluids. Regarding the assessment of siRNA association within the formulation, severe drawbacks, particularly lack of consistency between replicates, hindered the successful determination of siRNA content. Moreover, major difficulties faced regarding the extraction of the aqueous phase after destroying the lyophilized nanoparticles proved to be an issue that needs to be improved. However, despite these handicaps, it was apparent that successful association of

siRNA with the formulation, even if lower than desired, could be observed. Furthermore, the impact of polyamines, namely spermidine, while not completely clarified, appears to be positive, as it seems to increase the signal obtained from the siRNA.

Finally, NPs formulations were incubated with CCR5-positive cultured cells, and seemed to, at least at some concentrations, decrease CCR5 expression. Particularly, formulations containing siRNA complexed with spermidine appear to exert a similar effect on CCR5 expression when compared with the same siRNA delivery through commercial transfection agents.

Overall, siRNA-containing PLGA-PEG-based NPs were successfully and consistently produced and characterized for colloidal properties. Despite the issues regarding siRNA quantification, these formulations present attractive features for potential development of anti-HIV microbicides. However, optimization of several processes, as well as a more reliable quantification method, are required.

Despite the mentioned conclusions of this work, several and important questions are still to be answered. For instance, the process of siRNA transfection into cultured cells utilizing commercial reagents offers room for optimization, namely testing time points other than 72 hours, as well as the possibility of repeating the transfection at 24 hours after the initial one, as reported previously [96]. Tuning these factors might lead to both more consistent outcomes and stronger CCR5 silencing, which would be crucial to better understand and more reliably compare with proposed siRNA delivery approaches. Another important aspect is the analytic technique used to quantify CCR5 expression. While FACS is attractive, mainly due to its simplicity and being a quantitative method, different techniques, such as immunodetection [155] or western blot [156], could also be explored. Furthermore, CCR5 quantification at the transcript, rather than protein, level is also an approach that has been pursued. Particularly, real time quantitative PCR (RT-PCR) is a technique that has been explored regarding this receptor quantification [157, 158], and could represent an interesting approach to be further studied.

Concerning the production of NPs, tuning of some parameters remains to be addressed. For instance, changes in poloxamer 407 concentration, use of different polyamines, other N/P ratios, and modifications regarding both the volume of the internal phase and the amount of siRNA incorporated might be sought in order to improve the obtained formulations, and strengthen their robustness. However, the biggest issue regards the incorporation of siRNA, particularly the attainment of unequivocal evidence that siRNA is indeed associated within the NPs. One interesting avenue worth pursuing might be the analysis of the siRNA-polyamine complexes before incorporation with the NPs, in order to better assess the attainment of the conjugates. In spite of that, the major hurdle to overcome is how to reliably quantify the amount of siRNA present in the nanoparticles suspension. While the commercial kit that was

used in this work did not yield consistent results, there are other options, such as PicoGreen or NanoDrop assays [6, 159], that can be explored.

Finally, several optimizations regarding the NPs incubation with CCR5-positive cells might be pursued. In particular, assessing the impact of higher concentrations, as well as comparing the effect of fresh and lyophilized NPs are important studies that should be included in future testing. Moreover, the safety of these formulations must be assessed, which can be achieved by multiple assays, such as the MTT reduction assay, lactate dehydrogenase leakage assay or the comet assay. Furthermore, interaction between NPs and CCR5 can be studied by microscopy, namely electronic and confocal, in order to assess the uptake of these formulation by the cells. Moreover, moieties that have been shown to specifically target CCR5, such as the D-Ala-Peptide T-Amide (DAPTA) [160], might lead to an increase in NPs uptake, and, subsequently, to stronger silencing effect. Several *in vitro* models relevant to mucosal delivery, such as monolayer models and multilayered cell constructs, could also be used to better mimic *in vivo* scenarios, offering insights regarding the possible behavior in biological context.

The proposed future work will be pivotal in ascertaining the general strategy followed in this work for engineering NPs promoting the silencing of CCR5. In case the proposed siRNA-loaded PLGA-PEG-based NPs are proven promising for advancing for further development, additional studies leading to stronger association of the siRNA within the formulation, as well as adequate targeting and posterior *in vivo* studies for assessing mucosal distribution, pharmacokinetics, safety and efficacy may be appropriate to pursue [161]. Conversely, alternative approaches to silence CCR5 expression or rendering this receptor useless for HIV entry in susceptible cells through specific inhibitors, such as maraviroc, may be considered appropriate.

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