Nanocarriers for systemic siRNA delivery to tumor vasculature

Afrouz Yousefi

2014

The printing of this thesis was financially supported by:
Enceladus, Amsterdam, The Netherlands
Utrecht Institute for Pharmaceutical Sciences, Utrecht, The Netherlands
Lipoid GmbH, Ludwigshafen, Germany

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Ph.D. Thesis, with a summary in Dutch

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Performing this research was supported by the Dutch Top Institute Pharma (project T3-301).
February 2014

ISBN
978-90-393-6101-6

Cover
Pendar Nabipour

Lay-out
Promotie In Zicht

Print
CPI Wöhrmann Print Service

Nanocarriers for systemic siRNA delivery to tumor vasculature

Nanodeeltjes voor de systemische afgifte van siRNA aan het tumorvasculatuur

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op maandag 17 maart 2014
des middags te 2.30 uur

door

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geboren op datum 19 september 1982
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“The world is full of magical things patiently waiting for our wits to grow sharper.”

- Bertrand Russell

Contents

1

General introduction

Cancer and angiogenesis

According to the data published by the International Agency for Research on Cancer of the World Health Organization, the risk of dying from cancer before the age of 75 is 11% worldwide in both genders [1]. Although most chemotherapeutics investigated so far aim at tumor cells, tumor vessels have also been suggested as interesting targets for therapy, especially in combination with chemotherapy [2], [3], [4]. Tumor blood vessels are the target of the therapeutic approach addressed in this thesis.

One of the hallmarks of malignancies is rapid cell division, which is dependent on availability of nutrients and oxygen. In order to provide sufficient supplies after the tumor size exceeds a critical size (~ 1-2 mm^3), tumor cells should be able to induce formation of new blood vessels, an event known as angiogenesis [5]. Angiogenesis is normally controlled by a balance between pro- and anti-angiogenic factors, but positive regulators are favored during the angiogenic switch [6], [7]. Anti-angiogenic therapeutics that limit vascularization are a more recent class of anticancer compounds. In addition to direct suppression of tumor growth by limiting supply of nutrients and oxygen to tumor cells, anti-angiogenic agents have been reported to normalize tumor vasculature at low doses which could improve delivery of chemotherapeutics in combination therapy [8], [9], [10]. Another benefit of anti-angiogenic therapy compared to conventional therapies is that it can be used against different tumor types. The target cells of an anti-angiogenic therapy, usually the endothelial cells of tumor vasculature, are readily accessible from the circulation and genetically more stable than tumor cells [11] and therefore less likely to develop drug resistance.

The main growth factors involved in angiogenesis include platelet derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), and angiopoietins. Among these, VEGF seems to be an attractive target for therapy, because it is arguably the best studied angiogenic pathway. The exact role of some of the other factors still needs to be better understood before entering the clinic (i.e. PDGF and angiopoietins) [12]. Also, unlike VEGF, some of these factors have an indirect effect on angiogenesis. For instance, binding of EGF to its receptor (EGFR) triggers angiogenesis indirectly through upregulation of pro-angiogenic factors such as VEGF. The critical impact of VEGF and its cognate receptors is more established than the other angiogenic factors making this pathway a main target in anti-angiogenic therapy [13].

VEGF is a regulator of vascular development and is crucial in angiogenesis. While VEGF refers to a family of at least seven isoforms, the term VEGF usually represents the VEGF-A isoform which plays the most prominent role in angiogenesis. VEGF-A can bind to receptor tyrosine kinases VEGFR-1 and VEGFR-2, whereas VEGF-C and VEGF-D bind to VEGFR-3 [14]. The exact function of VEGFR-1 and

VEGFR-3 are still unknown but they are implicated in migration of monocytes and macrophages and lymphangiogenesis, respectively [12]. VEGFR-2 regulates events related to vascular endothelial cells. Phosphorylation of VEGFR-2 upon interaction with VEGF activates a series of signaling cascades leading to pro-angiogenic events such as cell proliferation and migration [15]. Therefore, inhibiting the production or activity of VEGFR-2, in particular, could be a valuable strategy in cancer treatment. This inhibition can be achieved at the level of protein, through small molecular weight inhibitors or monocolonal antibodies, and also at the level of mRNA by nucleic acid based approaches, such as RNA interference.

RNA interference in tumor anti-angiogenic therapy

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism which involves complementary base-pairing of small RNAs with the target mRNA leading to regulation of gene expression. Small interfering RNA (siRNA) is the most extensively researched small RNA for therapeutic purposes which has entered several clinical trials [16]. This small double-stranded non-coding RNA is composed of 21-23 nucleotides and can be generated synthetically to be used for clinical applications. The mechanism of action of RNAi by siRNA begins when double-stranded RNA of exogenous origin with a length >21 nucleotides is cleaved by an RNase type III enzyme Dicer. Importantly, since long double-stranded RNAs spark an immune response intracellularly, the maximum length should be <30 nucleotides. The product of this cleavage is a duplex of an antisense (guide) strand and a sense (passenger) strand with two nucleotide overhangs at the 3´ position. Some approaches use the mature 'cleaved' siRNA product from the start. This duplex is incorporated within the RNA-induced silencing complex (RISC) in association with Argonaute proteins. Thereafter, the sense strand is degraded and the activated RISC continues to direct the antisense strand to the target mRNA [17], [18]. Depending on the level of complementarity between small RNA and mRNA, the mRNA is cleaved and degraded or the translation complex is disturbed and protein translation is stalled.

Since the discovery of RNA interference (RNAi) in 1998 by Andrew Fire and Craig C. Mello in the nematode *Caenorhabditis elegans* [19] and later in mammalian cells [20], considerable research has been directed towards evaluating the therapeutic potential of this phenomenon. In anti-angiogenic cancer therapy, siRNA offers a different approach compared to monoclonal antibodies and small molecule inhibitors which are commonly used. A suitable siRNA candidate with anti-angiogenesis outcome is siVEGFR-2 (with the sequence of sense strand: 5´-GGA-AAU-CUC-UUG-CAA-GCU-AUU-3´ and anti-sense strand: 5´-UAG-CUU-GCU-AGA-GAU-UUC-CUU-3´) which would silence expression of VEGFR-2 in endothelial cells of

tumor vasculature and thereby interfere with angiogenesis as described in the previous section.

siRNA delivery

Despite the fact that siRNA is attractive as a therapeutic, several challenges need to be solved to achieve successful therapy. Although local administration of siRNA has been reported to induce gene silencing [21], not all tumors can be reached via this method and also, this method of administration cannot be applied to micrometastatic disease. These limitations could be overcome by systemic delivery. However, naked siRNA administered systemically is degraded easily by serum RNase A-type nucleases and is rapidly cleared by the kidneys [22]. Moreover, the fact that siRNA is a highly anionic large molecule impedes its interaction with target cell membranes and its cellular internalization [23]. Targeted carrier systems can help maintain integrity of siRNA in the circulation as well as facilitate uptake by target cells. Viral and non-viral carriers have been studied for delivery of siRNA. In this thesis we have focused on the non-viral group, which are thought to impose less safety concerns than viral vectors.

Amongst a plethora of different carrier systems being developed for siRNA delivery, polymeric and lipidic nanoparticles are the two main classes of carriers under investigation [24], [25], [26], [27], [28]. Efficacy of carriers depends on being able to cross extracellular and intracellular barriers in the body. The first barrier that intravenously-injected nanoparticles encounter is the blood where interactions with serum proteins can occur. Opsonin proteins are readily absorbed on the surface of foreign particles which would facilitate recognition by the mononuclear phagocyte system and would thereby accelerate clearance. As such, an important feature of an optimal delivery system is that it would prevent opsonization. PEGylation has been predominantly used to reach this goal, by shielding carrier systems in the circulation and creating a stealth effect [29]. PEGylated nanoparticles have been reported to possess longer circulation half-lives. Nevertheless, applying PEGylation needs to be tuned in a way that it does not compromise cell uptake and endosomal escape [30]. Among the influential factors determining the fate of nanoparticles in the blood are interactions with components of the complement system, platelets, and immune cells which would call for performing hemocompatibility studies on formulations.

Apart from the blood, the complex extracellular tumor matrix, the target cell membrane and the endosomal membranes of target cells are additional hurdles to be overcome before siRNA can reach its site of action in the cytosol. Regarding interactions with the plasma membrane, targeting strategies may improve cellular internalization of nanoparticles. Active targeting facilitates cellular internalization by

receptor-mediated endocytosis. A common active targeting strategy in cancer therapy has been attaching a peptide harboring an RGD motif to polymers, lipids, and peptides [31], [32], [33], [34]. RGD binds to integrin $\alpha_v\beta_3$ present on tumor endothelial cells and tumor cells which could aid in internalization of nanocarriers. In this thesis we have investigated a novel ligand, called anginex, which has not yet been studied for siRNA targeted delivery to tumor angiogenic vasculature. Anginex is a 33-mer peptide which has already been shown to bind to the galectin-1 receptor overexpressed on endothelial cells of tumor vessels. This interaction on itself has already been demonstrated to result in anti-angiogenic effects [35], [36]. Galectin-1 has a role during the angiogenesis process in endothelial cell adhesion to tumor cells [37]. Thus, in addition to the use of anginex as targeting ligand, also these effects could potentially be exploited (dual action strategy).

Objectives and outline

This thesis focuses on non-viral delivery systems of siRNA for systemic administration. We have studied lipoplexes with the composition of DOPE, CHEMS, DSPE-PEG2000, DSPE-PEG2000-Mal) containing siVEGFR-2. These lipoplexes are targeted to galectin-1 by being coupled to the peptide anginex to suppress tumor angiogenesis. We have investigated the hemocompatibility of this system alongside a polymeric nanocarrier (Poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol (pHPMA-MPPM)-based polyplex) and have introduced a method for post-insertion of DSPE-PEG2000 into pHPMA-MPPM polyplexes with the aim of decreasing their interactions with blood components.

In **Chapter 2**, an overview is given of the biological barriers that polymeric nanoparticles encapsulating DNA or siRNA are facing after intravenous administration. This overview is used as a guide towards designing *in vitro* assays which are representative for the *in vivo* situation. Moreover, the relevance of *in vitro* assays for translating results from *in vitro* to *in vivo* is discussed and implications for DNA and siRNA delivery are highlighted.

In an attempt to decrease the positive surface charge of pHPMA-MPPM-based siRNA polyplexes temporarily, a physical method for post-insertion of DSPE-PEG2000 is studied in **Chapter 3**. In this method, DSPE-PEG2000 is inserted non-covalently to the particles after the formation of polyplexes. The impact of post-insertion on physical properties, carrier unpacking, and cellular toxicity of pHPMA-MPPM polyplex is investigated.

Chapter 4 deals with the possibility of using galectin-1 targeted anginex lipoplexes (Angiplex) containing siVEGFR-2 for anti-angiogenic therapy. This chapter aims to observe the gene silencing efficiency in endothelial cells *in vitro*. First,

Angiplex is characterized physicochemically and its cellular toxicity is evaluated in human umbilical vein endothelial cells (HUVECs). Then the biological effects including cell association, internalization, and gene silencing activity of this system are investigated using HUVECs.

In **Chapter 5**, the hemocompatibility of Angiplex and pHPMA-MPPM-based polyplexes containing siRNA have been compared to demonstrate the impact of physicochemical characteristics of delivery vehicles on their interactions with blood components, blood cells, and the immune system. Thrombogenicity, activation of the contact system in coagulation, complement activation, and TLR stimulation have been investigated to help predict the behavior of these particles in the blood *in vivo*.

To investigate the *in vivo* fate of Angiplex containing siVEGFR-2, which was evaluated *in vitro* in Chapter 4, the biodistribution and anti-angiogenic tumor suppression efficacy of this system are studied in a Neuro-2A tumor model in A/J mice in **Chapter 6**. In the biodistribution study, the presence of nanoparticles in plasma is detected by Stem Loop-Reverse Transcription (SL-RT) PCR of siVEGFR-2 at different time points and the same method is used to determine the accumulation in tissues including the tumor tissue. In the efficacy study, the tumor size is monitored.

Chapter 7 presents a summary of the findings obtained in this thesis, and provides additional discussion and perspectives.

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2

Trends in polymeric delivery of nucleic acids to tumors

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J. Control. Rel. 170(2):209-218 (2013)

Abstract

Delivery of nucleic acids to tumors has received extensive attention in the past few decades since these molecules are capable of treating disease by modulating the source of abnormalities. Although high efficiency and low toxicity of numerous delivery systems for nucleic acids has been approved frequently with *in vitro* assays, contradictions have been observed in many cases between these results and what has occurred in the dynamic *in vivo* situation. Filling this gap seems to be crucial for further preclinical development of such systems. In this paper, we discuss various barriers which polymeric DNA or siRNA nanoparticles encounter upon systemic administration with an aim to assist in designing more relevant *in vitro* assays. Furthermore, individual considerations concerning delivery of DNA and siRNA have been addressed.

1. Introduction

Nucleic acids (NA) are attractive therapeutics. They promise specific interference at the root of the disease, have the possibility to address undruggable targets, and can be adapted to different diseases by simply changing the nucleotide sequence. At present, the reality is that NA are expensive and that their application still lacks safe and efficient delivery systems. As a result, most attention is focused on life-threatening diseases such as cancer where smaller risk-benefit ratios are accepted. These efforts have led to a multitude of articles describing the *in vitro* efficacy of nanocarriers for delivery of NA.

However, despite this avalanche of *in vitro* data, only few carriers have shown promising results *in vivo*, mainly due to a poor translation of these systems from an *in vitro* testing environment towards the *in vivo* situation [1], [2], [3], [4]. *In vitro* optimizations have failed to simulate *in vivo* requirements; particularly in creating a dynamic system with an extracellular transport phase where particles may aggregate and be taken up by macrophages and where molecular NA and carrier species may be cleared before arrival at the target cell surface. Only this last step is usually studied *in vitro*. In addition, similar nanocarriers are often employed for DNA or siRNA delivery based on similarities in charge, vulnerability to degradation, and inability to spontaneously cross the cellular membrane. However, specific characteristics of DNA and siRNA, most notably regarding molecular weight and site-of-action call for tailored carriers for each [5].

Delivery systems for NA can be classified in three main groups:

1. Polymeric systems in which NA is condensed and/or complexed through charge interactions between the positive groups of the polymer and the negative NA [3], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15], [16], [17], [18], [19].
2. Lipidic systems in which cationic lipids interact with negatively charged NA and complex with or encapsulate them [20], [21], [22], [23] and hybrid systems in which NA are complexed with cationic polypeptides and liposomes to form lipoplexes [24], [25].
3. Inorganic nanomaterials such as gold nanoparticles which form micelles [26] or possess cationic charges, that further assist in loading NA [27], [28]. Also, silica particles have been discovered which form complexes through electrostatic interactions [29], [30] and can precipitate with pre-formed polyplexes [31].

To shed light on factors influencing the translation from *in vitro* to *in vivo*, this paper gives an overview of the different barriers that polymeric delivery systems for DNA or siRNA need to cross. These barriers are encountered upon entering the body until reaching the target site. In this review, the required biological and technological considerations for delivery systems of siRNA and DNA are highlighted.

2. NA delivery; Barriers to cross

2.1. Barriers influencing behavior of carriers encountered *in vivo*

2.1.1. Transport barrier

Depending on the route of administration, the first barrier that therapeutic particles encounter could be skin, peritoneum, muscle, cerebral fluid, respiratory tract or the blood. Unless local delivery is intended, particles need to pass these barriers and reach the blood circulation in order to access the target site. Components of the extracellular matrix in the above-mentioned anatomical locations can easily interact with positively-charged DNA or siRNA complexes preventing them from reaching the target cell [32]. The focus of this review is on the intravenous route because firstly, this route provides the means to access disseminated tumors through the body and secondly, the current unresolved challenges in intracellular fate of NA call for tailoring their intracellular delivery before seeking alternative routes of administration.

Upon entering the bloodstream, any circulating foreign particle is prone to internalization by the mononuclear phagocyte system consisting mainly of kupffer cells of the liver and macrophages of the spleen. Recognition by these cells is facilitated via opsonins which are serum proteins binding to foreign particles [33], [34]. In an attempt to decrease phagocytosis and improve circulation half-life of particles, different strategies have been developed. For instance, steric stabilization has been achieved by surface-modification with hydrophilic molecules such as polyethylene glycol (PEG). PEG's action is double-edged. On the one hand, it increases stability of particles in blood by shielding them and reducing opsonization [33], [35], [36], [37] and, on the other hand, it reduces transfection efficiency by compromising the interaction with target cells [38], [39]. The latter attribute can be tackled through fine-tuning of PEG density [39] or using sheddable PEG by which the cleavage of the chemical bond between PEG and the carrier occurs in response to external enzymes (such as matrix metalloproteinases (MMPs) in the tumor environment [40]) or to the pH drop in the endosomes [16], [41].

It has been reported that the degree of PEG shielding depends on the PEGylation method since it influences the PEG corona around the particle. This is supported by research done by Verbaan *et al*., in which it is shown that post-PEGylated pDMAEMA DNA polyplexes lead to higher transfection efficiency *in vitro* and longer circulation time *in vivo* than their pDMAEMA-*graft*-PEG counterparts.

Another observation concerning PEG, which needs to be taken into account is that adsorption of blood proteins to PEGylated particles differs depending on the type of protein. Gaucher *et al*. have demonstrated that although PEG promotes adsorption of poly(D,L-lactide)-based nanoparticles to lower molecular weight proteins *in vitro*, it leads to longer circulation half-lives compared to poly(*N*-vinyl-pyrrolidone)-coated particles [2]. This could be due to the fact that clearance of

particles depends on the nature of the plasma protein with which they interact. As reported by Crielaard *et al.* apolipoprotein E absorbs liposomes sigificantly higher than albumin, α2-macroglobulin, β2-glycoprotein I, and fibronectin [42].

Regarding toxicity of PEGylated polyplexes, inconsistencies exist between *in vitro* and *in vivo* data. For instance, Meyer *et al.* have reported substantially higher toxicity of PEG-PLLDMMAn-Mel-siRNA polyplexes *in vivo* compared to *in vitro* [3]. One possible explanation would be that cytotoxicity *in vitro* is usually represented by cell viability while *in vivo* it could occur due to particle aggregation, occlusion, and lack of targeting moieties.

Above examples question the predictive value of *in vitro* experiments for the *in vivo* situation. This discrepancy between *in vitro* and *in vivo* shows the need for predictive *in vitro* assays. One such assay has been reported by using surface plasmon resonance (SPR). In this study, positive correlations have been found between pharmacokinetics of liposomes and their interactions with blood proteins which could help predict behavior of liposomes in blood circulation [42]. Such assays could reduce unnecessary *in vivo* studies and thus limit the number of animals used as well as save time and expenses.

2.1.2. Tumor targeting; extravasation at the right time and place

If the tumor cells are the target for therapeutic intervention, complexes should pass through the vascular endothelium after reaching the tumor via the systemic circulation. Specific properties of the target organ can be beneficial for the timely extravasation of particles. For instance, the enhanced permeability and retention (EPR) effect can be exploited for passive targeting to tumors. Vasculature in tumors differs from healthy tissue by being more permeable, having wider fenestrations of endothelial cells, less or even absent basement membrane, and less coverage by pericytes or smooth muscle cells. Tumors are also known to have a different lymphatic drainage [43], [44], [45], [46], [47]. These characteristics have two main consequences. Firstly, they lead to a higher vascular permeability as compared to healthy tissues which is favorable for carriers of a certain size (<200nm) and molecular weight (>40kDa). Secondly, they result in the intratumoral retention of particles for longer periods than in normal tissues which is advantageous for cellular uptake [47]. If drug molecules have a low molecular weight, they would diffuse readily in and out of the tumor whereas for nanoparticles diffusion is negligible and the driving force for transport is convection. When exploiting the EPR effect for enhancing the delivery of nanocarriers, it should be taken into account that the maximum accumulation of nanoparticles in tumors via the EPR effect is often lower than 10% and the majority of intravenously injected nanoparticles end up in spleen and liver. To take maximum advantage of the EPR, nanoparticles require to be circulating in blood for a long time in order for the EPR effect to be successful. Grafting water-soluble polymers such as PEG onto the particles is one way to reach this purpose [33], [35], [36], [37].

Delivery to tumors is dependent on their vasculature. Therefore, the balance between pro- and anti-angiogenic factors should be considered when anti-angiogenic drugs are used in combination with cytostatics. It has been suggested that anti-angiogenic therapeutics (such as antibodies targeted against vascular endothelial growth factor) normalize tumor vasculature at low doses which facilitates delivery of chemotherapeutics. At higher doses, however, there is less chance of delivery because of vast vessel disruption. Therefore, the vascular normalization window within which there is sufficient access of drugs to tumor cells should be considered in combination therapy of anti-angiogenic agents and cytostatics [48], [49].

After extravasation, nanoparticles encounter tumor interstitium which influences the transport of particles. The interstitium is a hydrophilic gel composed of collagen networks including hyaluronate and proteoglycans. Therefore, the physicochemical characteristics of nanoparticles determine their transport across this matrix. For instance, cationic particles traverse the tumor interstitium faster than neutral or anionic particles due to charge interactions [50]. In addition, interstitial pressure in tumors is higher than in normal tissues because of leaky vasculature and impaired lymphatic drainage in tumors. This hypertension hinders the convective transport of particles from the blood stream to the interstitium [48] but increasing the systemic blood pressure has been shown to improve this transport. Greish *et al.* have demonstrated that the EPR effect can be accentuated by means of vasoconstrictors such as Angiotensin-II (AT-II) or NO releasing agents such as nitroglycerin. The underlying reason is that hypertension caused by vasoconstrictors in normal tissue is restored to the normal blood pressure, while in tumor tissue this autoregulatory mechanism is lacking, due to the absence of smooth muscle layer [51]. As a result, higher blood flow, increased EPR effect and thus increased drug delivery in the tumor tissue have been observed for nanoparticles [51], [52], [53]. Inhibition of tumor growth has been reported when AT-II has been administered in combination with SMANCS/Lipiodol in various metastatic cancers [12]. Another vasoconstrictor which has been described to positively influence the EPR effect is nitroglycerin which produces NO and promotes tumor suppression when administered in combination with anticancer drugs [54].

2.2. Barriers influencing behavior of carriers encountered at the cellular level

2.2.1. Cell membrane

Small molecules with proper physicochemical characteristics readily pass cell membranes and show high availability if they are released from their carrier. NA-carrier complexes, however, are chemically less stable and should stay intact until reaching their intracellular site of action. Once particles arrive at target cells, they can attach to them and be internalized via one of the following routes:

2.2.1.1. Non-specific interactions

2.2.1.1.1. Cationic and PEGylated polyplexes and lipoplexes

Cationic polyplexes interact initially with the extracellular negative charge of sulfated proteoglycans which limits their transport in the interstitium. Taking into account that proteoglycans are highly anionic, the often cationic polyplexes are internalized mainly through charge interactions which leads to non-selective delivery and thus increased side-effects [55]. PEGylation seems to be a viable option to overcome adsorption-mediated uptake. It should be noted that for PEGylated particles lower uptake is achieved since they interact less with the cell membrane due to steric hindrance.

One strategy to improve the uptake of these particles has been to introduce detachable PEG to circumvent hampering effects of the hydrophilic PEG layer. PEG shedding has been achieved by thiolytic [56] or hydrazone cleavage [22], [57]. Nie *et al.* reported that lipoplexes with a hydrazone linkage to PEG showed a 40-fold increase in transfection efficiency compared to their stable PEGylated counterparts [22].

Furthermore, MMPs, which are highly expressed in certain tumor tissues, have been exploited to detach PEG from a multifunctional envelope-type nano device (MEND). In this study, PEG has been attached to DOTAP-DOPE-Cholesterol-pDNA complexes through MMP-sensitive bonds in the form of PEG-peptide-DOPE (PPD). Comparison between the *in vitro* behaviour of covalently-bound-PEG MENDs and PPD MENDs has indicated an MMP concentration dependent and PEG density dependent transfection activity which has been higher in all cases for PPD particles. *In vivo*, however, stability of PPD particles has been shown to be low in blood circulation. In order to benefit from both covalent-PEG's effect in prolonging half-life and positive properties of PPD on transfection activity, 1:1 mixtures of covalent-PEG and PPD have been attached to MENDs which has successfully resulted in higher transfection activity *in vivo* [40].

2.2.1.1.2. Polyplexes containing cell-penetrating peptides

Cell penetrating peptide (CPP)s are short cationic or amphipathic peptides internalized via endocytosis [58], [59], [60], [61] or direct membrane penetration [62], [63], [64]. The exact mechanisms involved in their uptake are still under investigation but it is believed that they form stable particles with NA and deliver them indirectly by assisting in the clathrin-dependent endocytosis of polyplexes [65].

The rate-limiting step for CPP-based vectors is endosomal escape which can be facilitated in various ways. To improve delivery by CPP vectors several tools are available such as conjugation with endosomolytic moieties like histidine-containing alpha-helical peptide stretches and cysteine residues [66], fusogenic peptides such as HA2 [67], [68] and stearyl derivatives of octaarginine and other amphipathic CPPs [69], [70], [71], [72], [73].

Since attaching CPPs covalently to nucleic acids has proven to be difficult, the group of Langel have proposed an alternative non-covalently bound complex of nucleic acids and a series of CPPs called PepFects and Nickfects. Complexes made from Pepfect14 and PepFect6 with siRNA have demonstrated efficient RNA interference *in vitro* and *in vivo* [74], [75].

Although CPP systems seem promising *in vitro* and locally *in vivo* [76], [77] there have not been many successful reports on their systemic *in vivo* administration due to side effects [78]. Specifically targeted CPPs are alternative options to overcome challenges *in vivo* by providing specific binding of the particles to target cells as shown by Kumar *et al.* [79]. In this study they have successfully delivered siRNA complexed with a short peptide derivative of rabies virus glycoprotein to neuronal cells of mice. Specific uptake has occurred via acetylcholine receptor which is widely expressed in neuronal cells.

2.2.1.2. Specific interactions using targeting ligands (active targeting)

Including targeting ligands in nanocarriers increases the chance of target cell interaction and internalization and it influences intracellular trafficking pathways. These ligands initiate receptor mediated endocytosis [80]. Numerous receptors overexpressed on tumor cells have been identified, namely folate receptors (FR), transferrin receptors (TFR), integrins, σ receptors, epidermal growth factor receptor (EGFR) and HER2 receptors. Small molecules and antibodies are two main ligand categories which have been investigated for targeting to these receptors.

2.2.1.2.1. Small natural ligands

Among small natural ligands, folic acid has received much attention since it can promote receptor-mediated endocytosis by certain tumor cells overexpressing folate receptors. Because of high affinity, low immunogenicity and low toxicity, folic acid has been used to increase transfection efficiency of poly(DMAEA-*co*-BA) phosphazene-based polyplexes [81] and cyclodextrin polyplexes [82], [83], [84]. Interestingly, it has been suggested that charge neutralization of folate grafted PEI600 cyclodextrin polyplexes by interaction with serum albumin enhances transfection efficiency *in vitro* as well as compatibility *in vivo* [83], [84]. For siRNA delivery, (oligo-ethanamino)amide-based polyplexes equipped with folic acid residues have been reported to lead to significant silencing efficiency *in vitro* and *in vivo* [85].

Targeting to TFR is another possibility which has been investigated by Bartlett *et al.* in NOD/SCID mice injected with Neuro2A-Luc cells. They demonstrated that tumor localization has been identical between transferrin-targeted and nontargeted cyclodextrin-polycation-siRNA complexes. However, luciferase knockdown was significantly improved when including the targeting ligand [86]. In a similar study, non-targeted and transferrin-targeted PEGylated cyclodextrin polymers were assessed

for DNA delivery in NMRI mice bearing HT-29 tumors. Nontargeted particles were detected in the tumor fibers but only targeted particles showed intracellular localization [87]. These results, have revealed that ligands might not have an effect on the target cell accumulation but they play a pivotal role in facilitating tumor cell internalization.

σ receptors have also been investigated as targets for polyplexes. By including anisamide (AA) in a DSGLA (*N*,*N*-distearyl-*N*-methyl-*N*-2[*N'*-(*N*2-guanidino-L-lysinyl)] aminoethyl ammonium chloride) and polycation-DNA complex, Chen *et al.* have shown considerable uptake in human lung carcinoma cells *in vitro* and enhanced tumor regression *in vivo* [9].

One step further in targeting would be to target multiple receptors at the same time. Nie *et al.* investigated a dual-targeted gene delivery system based on PEG-PEI equipped with both RGD and B6 peptides targeting integrins and TFR, respectively. They reported higher transfection efficiency of dual-targeted versus single-targeted polyplexes due to the synergistic effect of these ligands. The RGD has acted as facilitator of cell association through integrins and B6 peptide as facilitator of endocytosis through TFR [23].

2.2.1.2.2. Antibodies

Monoclonal antibodies (mAbs) against certain receptors overexpressed in tumor cells have been quite attractive as targeting ligands during the last few decades. For instance, the tyrosine kinase epidermal growth factor receptor (EGFR) is a member of the ErbB family which promotes tumor cell proliferation, angiogenesis and metastasis. Monoclonal antibodies targeting EGFR (9B9 mAb) complexed with poly-ethylenimine-grafted-a,b-poly(N-3-hydroxypropyl)-DL-aspartamide (PHPA-PEI) pDNA polyplexes have enhanced transfection efficiency in cells overexpressing EGFR *in vitro* compared to non-targeted polyplexes [18]. In the same study, inhibition of tumor volume growth by targeted polyplexes has been achieved in a human hepatoma tumor model (SMMC-7721) in Balb/c nude mice.

Trastuzumab is another monoclonal antibody with a different target (HER2 receptors) and has been studied as conjugated to PEG-PEI polyplexes by Germershaus *et al*. They have observed improved delivery from two main aspects when including Trastuzumab. Firstly, it has increased transfection efficiency by active targeting. Secondly, less erythrocyte hemolysis and aggregation *in vitro* has occurred in case of Trastuzumab conjugated polyplexes due to their lower zeta-potential than unmodified PEG-PEI polyplexes. Moreover, since positively charged particles are known to activate the complement and promote uptake by the liver and spleen [88], [89], charge reduction by Trastuzumab could lead to lower complement activation and thus improve pharmacokinetics [90].

Although the above examples seem promising for targeted delivery, complexity and high production costs of mAbs have impelled the search for alternative molecules.

Also, attaching antibodies to nanoparticles would lead to increased risk of toxicity since antibodies have been shown to induce immune response in patients [91], [92]. Fab fragments of antibodies are simpler and more affordable candidates. Developments in phage display have provided a high throughput means to generate recombinant antibodies and select for specific fragments [93], [94]. In addition, Fab fragments are expected to be less immunogenic than whole antibodies because the Fc portion of antibodies is responsible for triggering antibody-dependent cellular cytotoxicity by reacting with Fc receptors on normal cells, macrophages, natural killer cells, and dendritic cells. However, Fab fragments have been shown to be quite unstable in the blood stream which has limited their application.

Nanobodies, which are single domain antibodies naturally produced in sera of camelids, could be the next plausible alternative. These molecules consist of the variable heavy chain domain of an antibody. Having a simple chemical composition which can be coded by a single gene facilitates their production and in fact after immunization of camelids, libraries of nanobodies can be made by simple cloning procedures. Moreover, since antibodies are complex molecules containing multiple disulfide bridges attaching the two subunits, yield of whole antibody production is low but nanobodies can be easily produced in higher amounts by cloning in bacteria. Other interesting features of these molecules are that compared to antibodies they show the same affinity and higher resistance to temperature and acidic or alkaline pH.

2.2.2. Endosomes

Several mechanisms are involved in the uptake of particles by cells as reviewed extensively by Bareford *et al.* [95]. Endocytosis, in which particles are engulfed within the cell membrane and subsequently enter the cell through vesicle formation, includes a complex range of pathways such as phagocytosis, clathrin/caveolin-dependent endocytosis, cell adhesion molecule-mediated endocytosis and macro-pinocytosis. After formation of endocytic vesicles, they may or may not fuse with lysosomes to enzymatically and hydrolytically degrade their contents. For NA particles which enter cells via the endolysosomal pathway, it is important to escape these vesicles on time to prevent early degradation. Studying endosomal escape routes of viruses can assist in designing systems with similar properties. It is not in the scope of this paper to give a comprehensive account of all the mechanisms involved in virus-host cell interactions. Therefore, examples are only provided as an overview and the reader is referred to the literature for more detailed information [96], [97].

It has been suggested that human adenovirus escapes endosomes by binding of its penton base to $\alpha_V\beta_5$ receptors on the host cells and subsequently activating these integrins. This activation has been directly related to membrane permeability and thereby endosome disruption [98].

With regard to the enveloped influenza virus, the hemagglutinin glycoprotein, present on the surface of the virus, is responsible for releasing it from the endosomes. The conformation of this protein changes under the acidic endosomal environment and causes membrane fusion [97], [99]. The HA2 subunit of hemagglutinin and diINF-7 derived from influenza virus have been utilized as fusogenic peptides to promote endosomal escape in gene delivery [89], [99], [100], [101], [102], [103], [104].

GALA is another example of a fusogenic peptide assisting in endosomal escape. It is a synthetic peptide in which glutamic acid is replaced by glycine in a mutant sequence of influenza virus. Efficient silencing *in vitro* and *in vivo* has been observed when in GALA-modified MENDs compared to non-GALA MENDs. These effects have been attributed to an accelerated endosomal escape triggered by GALA [11].

Several studies have been focusing on utilizing the intrinsic endosmolytic properties of the carrier itself. Polyethylenimine (PEI) is a well-known cationic polymer which disrupts endosomes by the so-called proton sponge effect: PEI causes pumping of protons into the endosomes due to its buffer capacity which leads to osmotic swelling and endosomal rupture [15], [105]. Moreover, coupling of hydrophobic moieties to PEI has been reported to facilitate endosomal escape through creating an even higher buffering effect [10]. Pun *et al.* have introduced an HIV-derived peptide (gp41) covalently bound to PEI to enhance its endosomal escape properties in HeLa cells [106].

Poly(amidoamine)s are a group of polymers used in DNA and siRNA delivery which seem to enable the same proton sponge effect. Although their mechanism of endosomal escape has not been clearly verified, a correlation has been observed between their buffering capacity and transfection efficiency [107].

2.2.3. Cytoplasm and nucleus

After reaching the cytoplasm, nanoparticles aimed for siRNA delivery should release their cargo. Upon arrival in the cytoplasm, the guide strand of siRNA binds to the RNA-induced silencing complex (RISC) which leads to target mRNA degradation and gene silencing. As shown by Xue *et al.* sustained release of siRNA-containing nanoparticles in the cytoplasm reduces cytotoxicity while keeping the biological activity of the siRNA intact. According to their results, acute and delayed cytotoxicity of sustained-release lipid-PEI siRNA particles was significantly lower than PEI siRNA polyplexes. The exact mechanism of this gradual release has yet to be clarified but it has been suggested that the gradual exposure of cells to the carrier plays a role in preventing toxicity [13].

For the delivery of DNA, a different type of nanocarrier is required which keeps the DNA protected during its residence in the cytosol. Both cytoplasmic and nuclear factors influence translocation to the nucleus. When designing vectors it should be

taken into account that low DNA mobility in the cytoplasm leads to low transfection. Viruses tackle this problem by interactions between viral capsid and the cytoplasmic microtubular network [108]. Suh *et al.* have found that microtubules are involved in active cytoplasmic transport of PEI/DNA complexes similar to that of adeno-associated viruses [109]. An important component which has been used as a tool in NA delivery is glutathione which is a common antioxidant agent in the cytosol. It has been reported to degrade polymeric carriers at the thiol group sites and therefore, facilitate efficient release of DNA and siRNA from polymeric transfectants [14], [17], [19]. Although nuclear localization ligands have been designed, there has been a good deal of evidence that the actual nuclear translocation of DNA occurs during mitosis [110], [111], [112], [113]. DNA can also enter the nucleus via specific subnuclear proteins which are part of the DNA transport machinery. NFkB protein complex has a nuclear localization signal peptide (NLS) moiety which stimulates nuclear pore opening. Vectors with several binding sites to NFkB show increased DNA uptake by the nucleus and inhibit the return of DNA to the endosome [114]. Van Gaal *et al.*, however, did not report any beneficial effects of DNA-targeting sequences on transgene expression [115]. In another study, Dean reported that import of pDNA to the nucleus depended on RNA-polymerase II as it could be prevented when activity of RNA-polymerase II in transcription was blocked. Not only is nuclear localization of DNA dependent on proteins in the cytoplasm, but it occurs also sequence-specifically only in the presence of SV40 enhancer [116]. Furthermore, cell-type dependent nuclear localization has been achieved by including sequences that can bind to specific transcription factors of the target cells [113], [117].

3. How to bridge *in vitro* and *in vivo* results?

From what is demonstrated in the literature, it is evident that the majority of work in NA delivery is performed *in vitro*. The impact of different formulation parameters and *in vitro* assays on the translation to *in vivo*, seems to be complex as demonstrated in a study done by Whitehead *et al.* They have found that for siRNA-containing lipidic nanoparticles, complexation method, entrapment efficiency and type of cell line used *in vitro* are correlated to the *in vivo* outcome while size (when diameter is less than 200 nm) and zeta potential of particles do not show a correlation [118].

It has already been reported that the type of cell line, the read-out systems and cell culture conditions have a significant effect on what you obtain *in vitro* [4]. These assays are generally tailored in a way to achieve high transfection efficacies rather than simulating the *in vivo* situation which hampers translatability between the two. Many examples exist from nanocarriers that lack toxicity and show good transfection efficiency *in vitro*, but are either quite toxic when injected into animals or do not show

the levels of transfection obtained in the rather artificial *in vitro* settings [119], [120]. For instance, poly(2-dimethylaminoethyl)methacrylate (pDMAEMA), a polymeric gene carrier, has been shown to generate high transfection activity *in vitro* in a study performed by Verbaan *et al*. *In vivo*, however, these particles have been accumulating mainly in the lungs [120]. One possible explanation would be that *in vitro* toxicity assays are mainly based on cell viability while aggregation, opsonization and uptake by mononuclear phagocyte system are the main players of *in vivo* toxicity. Moreover, immune-related toxicity may not be detected *in vitro* but may cause acute toxic effects *in vivo*. This poor *in vitro-in vivo* translation hampers preclinical development of such drug delivery system which is why we suggest that focus should be directed more on the development of better *in vitro* assays with predictive value for the *in vivo* situation. Some possible ways to achieve this goal are presented below.

3.1. Assays evaluating opsonization by blood proteins

Since opsonization influences clearance of nanoparticles *in vivo* to a great extent, assays evaluating opsonization by blood proteins can help scan for formulations with less binding to blood proteins at an earlier stage before entering *in vivo* experiments. This concept has already been investigated by Crielaard *et al.* where they have found correlations between binding of nanoparticles to five main opsonins (albumin, α2-macroglobulin, β2-glycoprotein I, apolipoprotein E, and fibronectin) and their blood clearance *in vivo* [42]. Taking this approach would save animals, costs, and time.

3.2. Aggregation/dissociation assays

As nanoparticles encounter blood components, they might aggregate which could cause blockade of microvessels. Moreover, they might dissociate which would render them ineffective. Size of particles is generally used as an indicator of these instabilities. Different tools such as Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA) are commonly used to characterize size of nanoparticles. When particles encounter plasma proteins their size increases which could be due to being encompassed by a layer of these proteins. Since DLS is based on detecting Brownian motion of an ensemble of particles it cannot show whether the sample is still monodisperse or not after mixing with plasma. NTA, on the other hand, has this capability since it tracks the particles individually. So it can confirm that no aggregates are formed and only the size of particles has increased in plasma and that the sample is still monodisperse [121].

In addition to NTA, other techniques have been introduced to test potential instabilities of nanomatter in biological fluids. A flow cytometry-based method has been developed for measuring size distribution of membrane vesicles, liposomes and DNA-containing nanoparticles that are fluorescently labeled in biological fluids.

The advantage of this method is that the presence of other particles such as serum components does not interfere with the analysis because fluorescence intensity is added as an inclusion criterion which helps distinguish between nanoparticle fluorescence and the background fluorescence from other components [122], [123]. Such method can be tailored for size distribution measurements in whole blood as well as determining ratio of cell-bound to free particles which would altogether provide an indication of their aggregation or dissociation.

Another method explored by Braekmans *et al.* to determine particle size and concentration is fluorescence single particle tracking (fSPT). In this method, the size of fluorescently labeled particles is measured in real time by imaging their diffusional motion with a fluorescence microscope in serum, plasma and whole blood. In their study, they have demonstrated that size distribution of liposomes in plasma and serum after injection to an artificial closed blood circulation system was not the same as in whole blood [124]. Therefore, methods which can be applied to whole blood seem more valuable in predicting the behavior of nanoparticles *in vivo*. In another related study, Nayae *et al.* observed rapid elimination of both free siRNA and siRNA encapsulated in dextran hydrogels after intravenous administration which suggested that the particles had dissociated partly immediately after injection. They tested this hypothesis with fSPT on plasma acquired from the same animals. The results showed an increase of particle size through time which indicated aggregation and therefore confirmed the partial stability of these particles [125].

3.3. Assays to test hematological effects

In vitro assays to assess the hematological effects of nanoparticles help predict potential *in vivo* toxicity issues. Hemolysis assay of nanoparticles is the first toxicity test to consider because a high volume fraction of the blood consists of erythrocytes. Particles with high hemolytic activity can induce severe consequences such as anemia [126]. Cationic particles seem to interact more with red blood cells than neutral particles and PEG-coated complexes [127]. Hemagglutination assays have also been used to assess degree of nanoparticle-induced aggregation of erythrocytes. Higher hemagglutination would indicate more occurence of microvessel occlusion. Platelets are other important blood components affected by toxicity of foreign particles with a major effect on the coagulation cascade and consequently thrombosis. Some studies have indicated a relationship between the activation of platelets measured by lumi aggregometry, transmission electron microscopy and flow cytometry to vascular thrombosis *in vivo* [128], [129] while correlations have been found between platelet aggregation and phagocytosis of foreign particles by macrophages [130]. In addition to the above factors, complement activation plays an important role in innate immune responses such as hypersensitivity reactions and anaphylaxis to foreign particles. Therefore, immunoassays evaluating levels of complement proteins can help screen

for nanoparticles with the lowest complement activation properties [126], [131], [132], [133]. As phagocytosis of particles by immune cells prevents them from reaching the target site, assays evaluating this phenomenon can provide valuable information regarding *in vivo* behavior of nanoparticles. Influence of formulation composition on phagocytosis has already been investigated by such an assay based on chemi-luminescence. Oxidative burst of polymorphonuclear cells after phagocytosis leads to production of peroxide radicals which can be quantified by this method as a measure of phagocytosis [134].

3.4. Blood flow simulation

One of the conditions absent in *in vitro* assays on cells is the flow of blood which could have an impact on the way cells react as well as on the clearance of nanoparticles from circulation. A transwell system is available (VI0.4 flow chamber: www.ibidi.de) in which a pulsatile flow of blood plasma containing the nanoparticles is pumped over cultured cells. This simulates fluctuations in the blood caused by the heartbeat. When a mix of phagocytic cells and target cells is cultured in such a system and exposed to nanoparticles under flow conditions, it has greater similarity to the *in vivo* situation than the static cell culture that is most often used. By fluorescently labeling the nanoparticles, their uptake in real time or their transfection efficiency can be measured by fluorescence microscope. Moreover, by assessing the concentration of particles in the flowing blood plasma, information about their clearance can be obtained which can help predict their circulation half-life *in vivo*.

3.5. Target tissue microenvironment simulation

Another area which could be explored more to fill the gap between *in vitro* and *in vivo* conditions is to simulate the complex network of interactions between different cells in the tumor microenvironment. *In vivo*, cell-to-cell communications occur either directly between cells or via molecular cross-talk. Three-dimensional (3D) co-culture systems (culturing two distinct cell lines) seem biologically more relevant than two-dimensional (2D) monocultures [135], [136], [137], [138], [139], [140], [141], [142]. In these systems, interaction of cells is taken into account in three dimensions. In addition, efficacy of drugs is usually overestimated in monocultures because uptake is easier in homogeneous cell cultures [138]. Taken together, 3D co-culture systems seem more representative of the dynamic *in vivo* situation. This concept was first introduced by Sutherland *et al.* who reported multicellular spheroids as an intermediate between 2D cultured cells and solid tumors [143]. These cell aggregates can be generated as heterogeneous cell populations in different layers representing various stages. The inner layer can become necrotic, the middle layer can be quiescent and the outer layer can consist of cells similar to 2D monocultures [144]. Moreover, 3D co-cultures are genotypically and phenotypically more similar to actual tumors as

reported in a study by Smith *et al.* where they use a Rotary Cell Culture System (RCCS™) to generate cell aggregates [145]. 3D multicellular aggregates can maintain their differentiated form better than monolayers as in the work of Shen *et al.* in which adipose-derived stromal cells have been reported to show higher levels of osteogenic differentiation once transplanted *in vivo* than cells cultured in monolayers [139]. Recently, Upreti *et al.* showed that galectin-1 expression after radiation increased with a similar pattern between co-cultured tumor-endothelial cell 4T1-2H11 cell spheroids *in vitro* and tumor-endothelial cell spheroids in mice. However, this pattern was not observed in 4T1 tumor cell only spheroids [140]. Another advantage of 3D cultured cells is that they have a more similar pattern of toxicity to *in vivo* than 2D cultured cells [141], [142].

3.6. Learning from natural vectors

Finally, one can learn from the mechanisms through which viruses attach to and penetrate host cells. Viral vectors have already been shown to be very effective although their applications have been restricted due to immunogenicity and safety issues. For instance, enveloped viruses consist of genetic material packaged by capsid proteins and enclosed by lipid bilayers. Some viruses attach to host cells by means of proteins present in the lipid bilayer and get internalized via clathrin-mediated endocytosis. These viruses have been used as a model for designing multifunctional envelope-type nano devices (MENDS) in gene delivery [21]. More specifically, the gene of interest has been complexed with a polycation (similar to a viral capsid) and later surrounded by lipidic bilayers containing the targeting ligand. Nuclear targeting has been achieved by using sugar moieties on the lipid bilayer [20]. These particles have shown significant improvement of transfection efficiency compared to non-modified MENDs.

In another study, higher transfection efficiencies were observed with dual-targeted polyplexes mimicking adenoviruses compared to single-targeted polyplexes. In this research, RGD and B6 peptide were attached to PEG-PEI polyplexes containing pDNA to target integrins and TFR. Their results demonstrated a biphasic uptake mechanism similar to viruses with RGD promoting cell binding and B6 peptide leading to endocytosis [23].

The same adenoviral approach has been taken to successfully deliver siRNA to human lung carcinoma cells *in vitro* and *in vivo* by a formulation containing anisamide, DSGLA (*N*,*N*-distearyl-*N*-methyl-*N*-2[*N'*-(*N*2-guanidino-L-lysinyl)] aminoethyl ammonium chloride) and DNA complexed to polycations. Anisamide residues have been used as targeting ligand to the overexpressed σ receptors on tumor cells [9].

Although clinical trials of NA therapeutics have been ongoing for two decades, few have proven to be successful in delivering these molecules systemically. Davies *et al*. have performed a phase I clinical trial on patients with metastatic melanoma to

deliver the M2 subunit of ribonucleotide reductase (RRM2) siRNA with a carrier consisting of a cyclodextrin-based polymer, PEG and transferrin ligand. In this study, RNA interference was confirmed at the mRNA level as well as the protein level in tumor biopsies. In addition, clear localization of particles in tumor cells and no side effects were observed [8]. Another study which is still ongoing, involves stable NA lipid particles (SNALP) [146], [147], [148].

When considering the above formulations which have found their way to clinical trials, one can note that shielded delivery systems similar to viruses in terms of having a cationic core and a targeting ligand might be the best candidates for NA delivery. Successful delivery of nanoparticles is generally achieved by improved pharmacokinetics and target site specificity. Nanoparticles approved for clinical trials possess enhanced circulation life time due to the hydrophilic polymer layer and show low toxicity partly because of highly specific ligands. Moreover, the cationic part of such carrier plays a promoting role in the unpacking step which is the bottleneck of NA delivery.

To design optimal delivery systems for NA one should consider all the stages that nanoparticles encounter since entering the body as well as study the structure of viruses and their invasion mechanisms. Moreover, given the poor *in vitro-in vivo* translation, it seems essential that better predictive *in vitro* assays are investigated and promoted in order to prevent unnecessary *in vivo* studies.

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3

Post-insertion of DSPE-PEG2000 in pHPMA-MPPM polyplexes

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Abstract

A major concern regarding nucleic acid carrier systems injected intravenously is to what extent they protect their cargo from the blood. Opsonin proteins in the blood cover foreign particles and facilitate their recognition by the mononuclear phagocyte system (MPS). Strategies aiming at reducing interactions with blood proteins have been mainly through tuning hydrophilicity and surface charge of nanocarriers. PEGylation is a prevalent method which interferes with interactions of nanoparticles and their surroundings through steric hindrance, shielding their surface charge and enhancing their hydrophilicity. However, prolonged circulation half life and including PEG moieties in the formulation are not always beneficial since they could lead to toxicity caused by anti-PEG antibodies, low cell uptake and limited endosomal escape. A strategy that may help overcome these limitations is temporary PEGylation. In the present study, a physical method of post-insertion of DSPE-PEG2000 to pHPMA-MPPM polyplexes encapsulating siRNA has been explored. We observed that post-insertion of DSPE-PEG2000 led to a decrease of ζ-potential in polyplexes at different N/Ps and reduced complex stability slightly. It was shown that interactions between DSPE-PEG2000 molecules and polyplexes plausibly occurred through interactions with the hydrophobic backbone of the polymer leading to an equilibrium state between the micelles and the post-PEGylated particles. Our findings also showed that post-PEGylated pHPMA-MPPM polyplexes induced less toxicity than non-PEGylated polyplexes *in vitro*. Such method of post-insertion could be convenient for temporarily introducing PEG molecules to already established nanoparticles in order to increase their circulation half life with reduced toxicity.

1. Introduction

One of the main properties of an optimal nucleic acid delivery system administered intravenously is extended half life in the blood which would increase encounter with target cells and improve their therapeutic effect. Since some plasma proteins tend to opsonize foreign particles and consequently lead to their recognition by the mononuclear phagocyte system (MPS), only nanoparticles which can escape this process would have a chance of reaching the target site. It is known that surface charge and hydrophilicity of particles play a key role in their interactions with blood components [1], [2], [3].

Nucleic acid carrier systems usually comprise of positively charged polymers or lipids which form particles with nucleic acids through charge interactions. However, positive charge increases clearance of nanoparticles by the MPS. PEGylation is an established method used to shield the surface charge of nanoparticles and to produce a stealth effect which both benefit circulation half life [4], [5], [6], [7]. Nevertheless, a long circulation half life would cause prolonged exposure of non-target organs to the particles and induction of anti-PEG IgM production [8], [9]. Moreover, PEG could hinder target cell interaction and endosomal escape [10]. Detachment of PEG from the particles assists with destabilization of endosomal membrane and its subsequent disruption. This dual effect of PEG has been described as the PEG dilemma [11].

Target cell interaction could be facilitated by attaching targeting ligands whereas production of antibodies and endosome stabilization can be prevented by de- PEGylation. For instance, Judge *et al.* demonstrated that a shorter alkyl chain in stabilized plasmid lipid particles decreased immune response due to anti-PEG antibodies because PEG molecules could diffuse more easily from the particles [12]. De-PEGylation has been mainly achieved by using sheddable/diffusible PEG [2], [8], [12], [13].

Although the majority of PEGylation has been performed through covalent attachment of PEG, from a production point of view, for established nanomedicine it could be challenging to change the formulation chemically. In addition, the PEG moiety also acts as an anchor for coupling of targeting ligands which usually have low stability in organic solvents and low solubility or are sensitive to stages of manufacturing. As such, it is attractive to search for alternative methods of PEGylation after the preparation of particles. Physical adsorption of PEG through charge interactions could be an interesting approach because on the one hand it is more convenient and could be applied after the preparation of particles. On the other hand, it provides the particles with different pharmacokinetic properties which would induce less immune toxicity [14]. Post-insertion of DSPE-PEG has already been investigated in liposomes, liposome-polycation-DNA complexes, and lipid nanocapsules with the aim of either incorporating a targeting ligand attached to DSPE-PEG or DSPE-PEG

itself [15], [16], [17], [18], [19]. The mechanism of post-insertion could be explained by properties of amphiphiles. In general, amphiphiles form micelles in aqueous dispersions and when these molecules possess a suitable balance between hydrophilicity and hydrophobicity, they could transfer from the micelles to phospholipid vesicles through co-assembly by mixing [20]. Although post-insertion of PEG-lipids into cationic complexes of siRNA and polymers seems counterintuitive, preliminary results in our lab suggested that this phenomenon took place. Therefore, in this study, we have further examined this concept for post-insertion of DSPE-PEG2000 in pHPMA-MPPM polyplexes encapsulating siRNA. PHPMA-MPPM is a biodegradable cationic polymer which has already shown successful silencing *in vitro* [21]. In this paper, the effect of post-insertion of DSPE-PEG2000 on physical characteristics, stability and cellular toxicity of polyplexes has been demonstrated and the mechanism of this incorporation has been investigated.

2. Materials and methods

2.1. Materials

Firefly luciferase siRNA duplex specifically targeting firefly luciferase (Fluc siRNA) was purchased from Integrated DNA Technologies BVBA (Leuven, Belgium) with the following sequence: sense strand 5′-GGUUCCUGGAACAAUUGCUUUUACA-3′, anti-sense strand 5′-GACCAAGGACCUUGUUAACGAAAAUGU-3′. Poly((2-hydroxy-propyl) methacrylamide 1-methyl-2-piperidine methanol (pHPMA-MPPM) with M_w of 240 kDa was synthesized and purified in our group as described previously [22]. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)] with PEG Mw of 2000 g.mol-1 was purchased from Lipoid (Ludwigshafen, Germany) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was obtained from Avanti® Polar Lipids Inc. (Alabaster, USA). Polyethylenimine (branched, M_w: 25 kDa) was supplied by Polysciences, Inc (Warrington, Pennsylvania USA). GelRed solution 10,000x in DMSO was purchased from Biotium (CA, USA). Cholesteryl hemisuccinate (CHEMS), heparin sodium salt from porcine intestinal mucosa and Nile Red were obtained from Sigma Aldrich (USA). Lipofectamine 2000 was obtained from Invitrogen (Breda, Netherlands). CytoTox-One Homogeneous membrane integrity assay was obtained from Promega Corporation (Madison, USA). All the other reagents were of analytical grade.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Verviers, Belgium). Endothelial cell growth medium-2 (EBM-2) supplemented with 2% FBS, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid,

0.1% hEGF, 0.1% GA-1000, and 0.1% heparin was purchased from Lonza (Walkersville, USA). HUVECs were cultured in EBM-2 completed medium in a humidified CO_2-incubator at 37°C. Experiments were performed on cells between passages 3-7 and mycoplasma tests were done regularly to confirm the absence of mycoplasma in cultures.

2.3. Preparation and physicochemical characterization of Fluc siRNA-pHPMA-MPPM polyplex, Fluc siRNA lipoplex, and DSPE-PEG 2000 micelles

Complexes of Fluc siRNA with pHPMA-MPPM at N/P ratios of 3, 5, 7, 10, 14, 20 and with PEI at N/P ratio of 8 were prepared. N designates the moles of cationic nitrogens in the polymer at pH 7.4 and P the moles of phosphate groups in siRNA. Polyplexes were prepared by adding Fluc siRNA solution (2.1 μM) to polymer solution in HEPES 20 mM, NaCl 150 mM (HBS buffer). The mixtures were gently vortexed for 5 seconds and incubated for 30 minutes at room temperature.

DSPE-PEG2000 aqueous micellar solution (1 mM) was prepared by dissolving the phospholipid in HBS buffer and incubation for 30 minutes at 60°C on rotavapor (Buchi, Switzerland) as described previously by Sato *et al* [23].

Lipoplexes were made by first dissolving DOPE, CHEMS, and DSPE-PEG2000 at molar ratios of 6:4:0.6, respectively, in ethanol:chloroform (4:3 v/v) on a rotavapor (Buchi, Switzerland) at 40°C. Fluc siRNA was complexed with protamine at molar ratio of 1:1.2 (w/w) in HBS and the mixture was incubated at room temperature for 20 minutes. After evaporation of the solvents under vacuum, the lipid film was hydrated by Fluc siRNA-protamine complex. siRNA concentration was 1 μM and lipid concentration was 10 mM. Repeated extrusion of the lipoplexes was performed by Lipex™ Extruder (Northern Lipids, Burnaby, BC, Canada) through polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) until a monodisperse sample was obtained with particles of approximately 100 nm.

Hydrodynamic diameter of particles was measured by dynamic light scattering at 25°C with a Malvern 4700 system using an argon-ion laser (488 nm) operating at 10.4 mV (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern Instruments Ltd., UK) with a measurement angle of 90°. Viscosity and refractive index of water at 25°C were applied. The system was calibrated with an aqueous dispersion of latex particles with a diameter of 200 nm (Thermoscientific, DE, USA). ζ-potential of polyplexes was measured at 25°C with a Zetasizer Nano-Z (Malvern Instruments Ltd., UK). The instrument was calibrated by polystyrene beads with known ζ-potential (Malvern Instruments Ltd., UK).

2.4. Post-insertion of DSPE-PEG2000 to pHPMA-MPPM polyplexes

Post-insertion was adapted from the method described by Perrier *et al.* [19]. Briefly, DSPE-PEG2000 aqueous micellar solution was added to polyplexes at micelle

concentration of 32 μM. The suspension was vortexed and incubated for 24 h at 60°C. Afterwards it was quenched on ice for 1 minute. Non-PEGylated polyplexes were treated under the same conditions and used as controls. Hydrodynamic diameter and ζ-potential were determined as described in section 2.3.

2.5. Agarose gel electrophoresis

Fluc siRNA pHPMA-MPPM polyplexes (N/P ratios of 7, 10, 14 with and without post-inserted DSPE-PEG2000) were prepared in HBS as described in sections 2.3. and 2.4. Polyplexes containing 60 pmol siRNA were applied to the slots of 4% (w/v) agarose gels and electrophoresis was performed at 100 V (constant voltage) for 45 minutes. siRNA bands were post-stained with GelRed according to the manufacturer's protocol. Bands were detected by UV transilluminator (ImaGo compact imaging system (B&L Systems), the Netherlands).

2.6. Heparin displacement assay

Fluc siRNA pHPMA-MPPM polyplexes at N/P ratios of 7 and 14 with and without DSPE-PEG2000 were prepared in HBS as described above in sections 2.3. and 2.4. Heparin was mixed with polyplexes at ratios of 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, or 0.15 IU heparin to μg siRNA. The mixtures were vortexed for 5 seconds and incubated for 10 minutes at room temperature before being applied to 4% (w/v) agarose gels. Electrophoresis and UV illumination were performed as described in section 2.5.

2.7. Nile Red assay

PHPMA-MPPM polyplexes were prepared at fixed N/P ratios (7 and 10) at varying siRNA concentrations (0.04, 0.08, 0.2, 0.4, 0.8, 2, 4, 8, 20, and 40 μM) and also at fixed polymer concentration (4.3 mg/ml, comparable with N/P:10) and varying siRNA concentrations (0.04, 0.08, 0.2, 0.4, 0.8, 2, 4, 8, 20, and 40 μM). In addition, pHPMA-MPPM solutions alone with polymer amounts identical to N/P:10 were prepared in HBS. Lipoplexes made as described in section 2.3 were diluted to the above siRNA concentrations and used as positive control and HBS was used as negative control. Nile Red assay was adapted from studies of Lim *et al.* [24] and Stuart *et al.* [25]. Briefly, 1.25 mM Nile Red stock solution was prepared in ethanol and mixed with the samples at ratio of 1:500 (v/v). The mixtures were stored overnight as room temperature and thereafter, fluorescence intensity was recorded with a Fluorolog fluorometer (Horiba Jobin Yvon, Japan) at 25°C. Excitation wavelength was 550 nm and fluorescence emission was measured from 550 to 700 nm. Log fluorescence intensity of Nile Red at 635 nm was plotted against log siRNA concentration. Results were presented as mean of three independent measurements ± SD.

2.8. LDH cytotoxicity assay

HUVECs were seeded in 96-well plates (4,000 cells/well) and cultured overnight. Polyplexes consisting of Fluc siRNA and pHPMA-MPPM at N/P:7 with and without post-inserted PEG (2, 16, or 32 μM) or Fluc siRNA and PEI at N/P:8 were prepared in HBS as described in sections 2.3. and 2.4. Particles were added to the cells at final siRNA concentration of 120 pmol/well in EBM-2 completed medium. The plate was incubated at 37°C for 4 h and CytoTox-One Homogeneous Membrane Integrity Assay was performed according to the manufacturer's protocol.

3. Results and discussion

3.1. Preparation and physicochemical characterization of Fluc siRNA pHPMA-MPPM polyplex and Fluc siRNA lipoplex

PHPMA-MPPM is a biodegradable cationic polymer (Figure 1) that can form stable complexes with DNA and siRNA [21], [22]. PHPMA-MPPM complexes with Fluc siRNA have been reported to possess notable silencing efficiency against luciferase gene expression in H1299 human lung cancer cells expressing firefly luciferase [21]. In this study, pHPMA-MPPM was complexed with Fluc siRNA at N/P ratios of 3-20. Polyplexes were in the size range of 100-250 nm with a PDI <0.3 and had an average ζ-potential of 22-45 mV (Figure 2). Size and charge increased gradually with the rise of N/P as indicated in Figure 2 which could be ascribed to the increase of positive charge due to the presence of more polymer in the higher N/Ps. Lipoplexes containing DOPE, CHEMS, and DSPE-PEG2000 at molar ratios of 6:4:0.6, respectively, encapsulating Fluc siRNA-protamine complexes had a mean size of 121 ± 3 nm (size ± SD) with a PDI of <0.2 and a mean ζ-potential of -8 ± 1 (ζ-potential ± SD). DSPE-PEG2000 micelles were also evaluated for their physicochemical characteristics and showed an average size of 14 ± 1 nm (size ± SD) with a PDI of <0.2 and ζ-potential of -5 ± 2 (ζ-potential ± SD). All particles were prepared in triplicates.

3.2. Post-insertion process and physicochemical characterization of PEGylated polyplexes

Post-insertion was performed at elevated temperatures to enable the exchange of PEG-lipids from the micelles into the polyplexes [26]. As the CMC of DSPE-PEG2000 micelles is low, post insertion was expected to occur slowly and therefore overnight incubation was performed. The effect of possible interactions between the micelles and polyplexes on diameter and charge of polyplexes at N/P:7-20 was further investigated. This range of N/P was selected because in gel electrophoresis studies, it was observed that at N/Ps lower than 7, the polymer-siRNA charge interaction was weak and thus the particles were not stable (see Supplementary Figure 1). Mean size

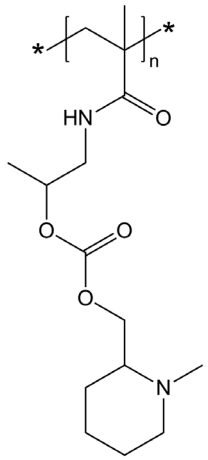

Figure 1 Chemical structure of pHPMA-MPPM.

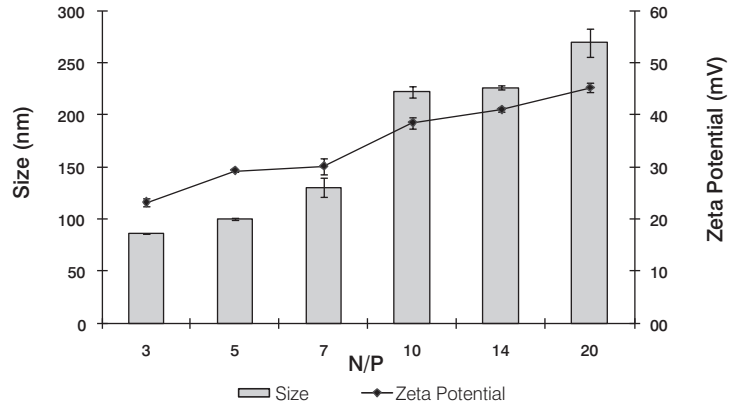

Figure 2 Size and ζ-potential of pHPMA-MPPM polyplexes with Fluc siRNA at N/P ratios of 3-20 (n=3).

of PEGylated polyplexes at N/P:7-20 (micelle concentration 32 μM) was 100-150 nm with a PDI of <0.4 while non-PEGylated particles were 130-250 nm with a PDI of 0.2-0.3 (Figure 3A) and micelles alone were 14 ± 1 nm (size ± SD) with a PDI of <0.2. The high PDI of the post-PEGylated particles indicated a polydisperse solution

containing polyplexes as well as free DSPE-PEG2000 micelles. Another observation was that the charge of complexes at N/P: 7, 10, 14, and 20 dropped 15, 6, 4, and 4 times upon inclusion of PEG, respectively. This could point to the possible post-insertion of DSPE-PEG2000 molecules leading to a shielding effect (Figure 3B). When N/P increased, the shielding effect seemed to be decreasing which could be due to the presence of more free polymer and thus more cationic polymer aggregates in the higher N/Ps.

It has already been reported in the literature that for surfactants with a high hydrophilic lipophilic balance (HLB) post-insertion is reversible. HLB of DSPE-PEG2000 was calculated according to Griffin's formula (HLB=20*M_w of PEG/M_w of the surfactant) to be 14.4 which would indicate high hydrophilicity [27], [28]. Taken this together with the results obtained in this section, it is likely that there is partial reversible incorporation of DSPE-PEG2000 monomers into the polyplexes in the form of an equilibrium from the micelle state to the polyplex-adsorbed state.

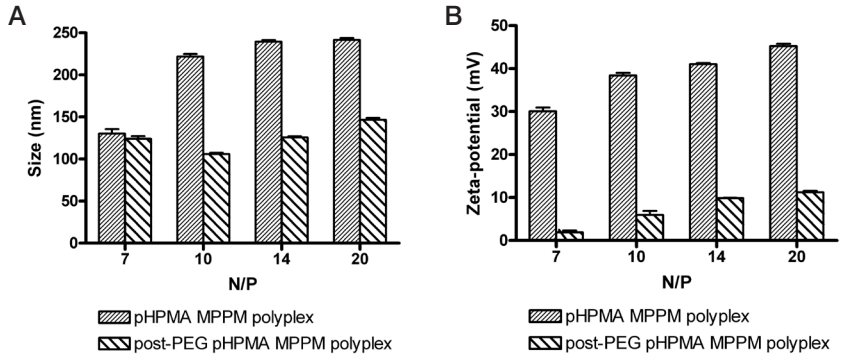

Figure 3 Comparison of size (**A**) and ζ-potential (**B**) of pHPMA-MPPM polyplexes and post-inserted PEG-pHPMA-MPPM polyplexes at N/P ratios of 7, 10, 14, 20 (n=3). Experiments were performed at fixed amounts of DSPE-PEG2000 micelles (32 μM).

3.3. siRNA is more strongly complexed with pHPMA-MPPM polyplexes than their PEGylated counterparts

Complexation of siRNA with pHPMA-MPPM at N/P: 7, 10, and 14 was evaluated by agarose gel electrophoresis. In presence of DSPE-PEG2000, siRNA was released more than non-PEGylated polyplexes which indicated lower stability after PEGylation (Figure 4; lanes 4-6 versus lanes 7-9). This effect could be attributed to interactions of

the hydrophobic tail of DSPE-PEG2000 amphiphiles with the hydrophobic areas of the polyplex which could disturb the integrity of the compact polyplex partially and thus facilitate siRNA liberation. siRNA release was the highest for PEGylated polyplex at N/P:7 likely due to less initial compaction of the particles compared to higher N/Ps because of lower cationic polymer amounts.

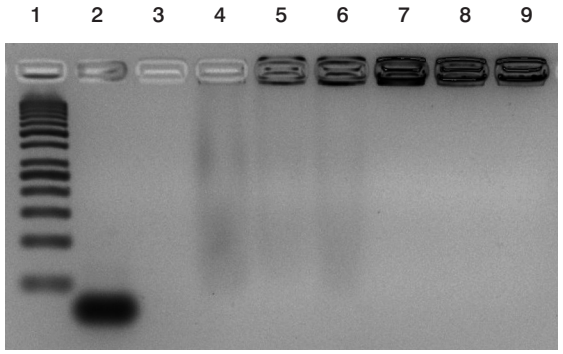

Figure 4 Gel electrophoresis of pHPMA-MPPM complexes with Fluc siRNA before and after post-insertion of DSPE-PEG2000. 1: marker, 2: naked Fluc siRNA, 3:pHPMA-MPPM alone, 4-6: post-PEGylated pHPMA-MPPM complexes with Fluc siRNA at N/P:7, 10, 14, respectively, 7-9: pHPMA-MPPM complexes with Fluc siRNA at N/P:7, 10, 14, respectively. siRNA concentration: 60 pmol/slot.

3.4. Post-PEGylation facilitates displacement of siRNA from pHPMA-MPPM polyplexes

To elucidate the effect of post-PEGylation on stability of pHPMA-MPPM polyplexes, displacement of complexed siRNA by heparin at different concentrations was investigated. PHPMA-MPPM polyplexes at both N/P:7 and 14 were stable after treatment with various concentrations of heparin (Figure 5A/5C; lanes 3-9) with the exception of 0.15 IU heparin treated polyplexes at N/P:7 (Figure 5A; lane 9). This could be a result of weaker charge interactions at N/P:7 compared to N/P:14 because of lower amount of polymer. However, as shown on panel B of Figure 5, heparin, which competes with siRNA due to its anionic nature, could replace siRNA in a concentration-dependent manner in PEGylated polyplexes at N/P:7. This is in line with the observation of Vader *et al.* where PEGylation also reduced poly(amido amine) polyplex stability against heparin displacement [29]. A more modest trend was observed at N/P:14 due to presence of more cationic polymer interacting with the

siRNA in the complex. It is generally accepted that nanoparticle stability should be tuned in a way that it offers sufficient stability in the blood to protect the nucleic acid from being degraded or cleared by the immune system. Furthermore, it is crucial that nanocarriers undergo degradation easily once in the cytosol in order to have therapeutic effect. Therefore, an ideal delivery system would require to have an intermediate stability which has previously been obtained by using sheddable/diffusible PEG [13], [8], [12], [2]. PHPMA-MPPM polyplexes have been reported to possess a high colloidal stability leading to their low transfection efficiency compared to PAMAM dendrimers [30]. From the gel studies presented here, we concluded that physical post-insertion of DSPE-PEG2000 could be a means to destabilize particles moderately similar to conditions created by sheddable/diffusible PEG. PEGylated pHPMA-MPPM polyplex at N/P:7 appeared to be fairly stable and therefore was chosen for further experiments.

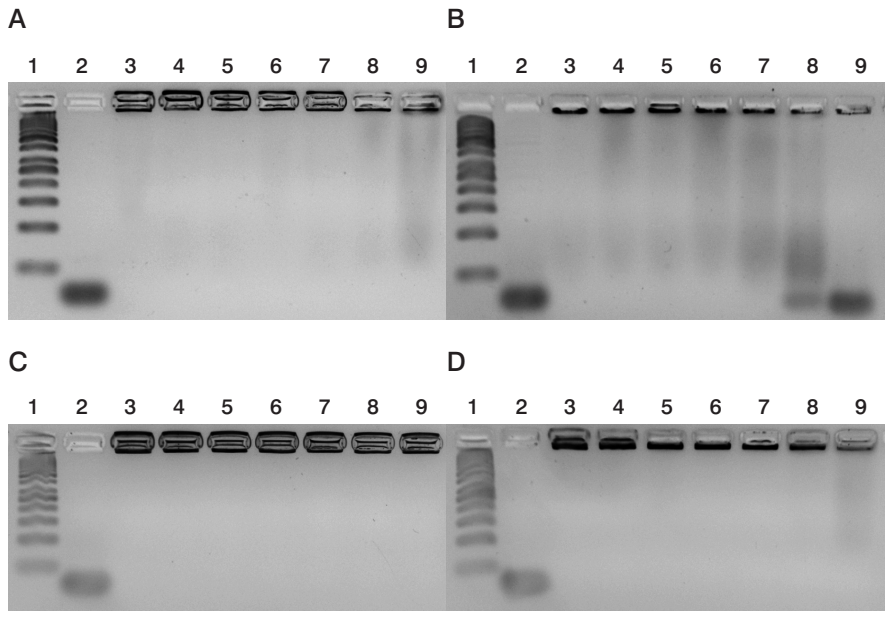

Figure 5 Heparin-induced dissociation of pHPMA-MPPM-Fluc siRNA at N/P:7 **(panel A)**, post-PEGylated pHPMA-MPPM-Fluc siRNA at N/P:7 **(panel B)**, pHPMA-MPPM-Fluc siRNA at N/P:10 **(panel C)**, post-PEGylated pHPMA-MPPM-Fluc siRNA at N/P:10 **(panel D)**. Lanes contain marker (lane 1), free Fluc siRNA (lane 2), 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.15 IU heparin per µg siRNA (lanes 3-9). siRNA concentration: 60 pmol/slot.

3.5. PHPMA-MPPM polyplex shows hydrophobicity

To assess the mechanism of interactions of DSPE-PEG2000 amphiphiles and pHPMA-MPPM polyplexes, Nile Red assay was performed. Nile Red is a fluorescence hydrophobic probe which fluoresces in different colors depending on the hydrophobicity degree of the medium in which it is mixed. As indicated in Figure 6, fluorescence intensity of Nile Red mixtures with pHPMA-MPPM polyplexes (N/P:7, 10) and pHPMA-MPPM polymer alone increased with particle concentration presented by siRNA concentration. However, when the concentration of siRNA was increased

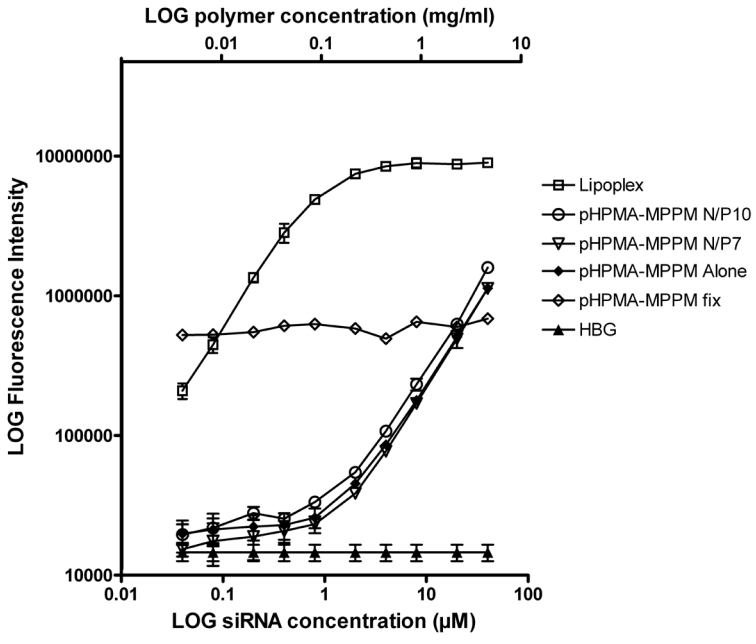

Figure 6 Fluoresence intensity after incubation of Nile Red with pHPMA-MPPM polyplexes at N/P:7 and 10 diluted to siRNA concentrations of 0.04, 0.08, 0.2, 0.4, 0.8, 2, 4, 8, 20, 40 µM; pHPMA-MPPM alone solution (4.3 mg/ml; equal to polymer amount at N/P:10); pHPMA-MPPM polyplexes with fixed concentration of polymer (N/P:10) and varying concentrations of siRNA: 0.04, 0.08, 0.2, 0.4, 0.8, 2, 4, 8, 20, 40 µM. All white symbols represent samples containing siRNA. Black diamonds represent samples containing no siRNA. Black triangles represent samples containing neither siRNA nor polymer. Excitation wavelength of Nile Red was 550 nm and fluorescence emission was measured in the range of 550 to 700 nm. Emmision at wavelength 635 nm is plotted (n=3).

and the concentration of polymer remained constant, no dose-dependent relationship was found (Figure 6; white rectangles). This implied that post-insertion had occurred because of interactions with the hydrophobic backbone of the polymer (Figure 1) and not the combination of siRNA and polymer creating a hydrophobic pocket. The distinction between DSPE-PEG2000 molecules bound to polyplexes versus those bound to free polymer remains to be elucidated.

3.6. PEGylation reduces cellular toxicity of pHPMA-MPPM polyplexes

Cytotoxicity was investigated by quantifying lactate dehydrogenase release as a measure of cellular membrane integrity using LDH assay. PHPMA-MPPM polyplex showed slightly lower toxicity than PEI polyplex which was used as positive control while LDH release for PEGylated pHPMA-MPPM polyplexes was about two-fold lower than non-PEGylated particles (Figure 7). These results revealed that the equilibrium between micelles and polyplexes shields the positive charge of the particles and thus limits their interactions with the negative cell membranes. There was no significant difference between different PEG concentrations which could be ascribed to the fact that micelle-polyplex interactions had already reached saturation. Micelle dispersion alone which was used as control exhibited low toxicity. One has to

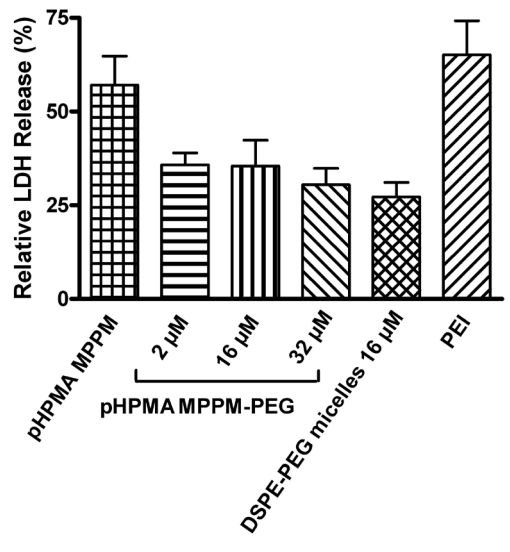

Figure 7 Cellular toxicity of PEGylated and nonPEGylated pHPMA-MPPM polyplexes at DSPE-PEG2000 concentrations of 2, 16, and 32 µM in HUVECs after 4 h incubation. LDH release represents toxicity. siRNA concentration: 120 pmol/well. Data are normalized to non-treated cells (n=3).

keep in mind that a high shielding effect could compromise uptake of polyplexes into the cells and consequently limit their efficiency. This refers to the fact that PEG shielding on the one hand gives stealth properties to the particles which benefits their circulation half life and on the other hand decreases particle-cell interactions and thereby efficacy. Therefore, when starting functionality assays, it is critical to tailor PEG amounts in order to find the balance between these controversial aspects and also to consider the use of a targeting ligand to improve uptake [11].

4. Conclusion

In this study, polyplexes of Fluc siRNA and biodegradable pHPMA-MPPM were prepared and were post-inserted with DSPE-PEG2000. Post-insertion of DSPE-PEG2000 resulted in decreased ζ-potential and increased polydispersity. It also reduced strength of interaction of the cationic polymer with the siRNA as shown in gel electrophoresis studies. Most importantly, post-PEGylation resulted in decreased cellular toxicity. When hydrophobicity of particles was tested, it was found that post-insertion was likely caused by interactions of DSPE-PEG2000 with the hydrophobic backbone of the polymer. From the data presented here, it can be suggested that the colloidal system investigated in this study is plausibly based on an equilibrium mixture of micelles and polyplexes which could be considered as a potential siRNA delivery system with more favourable properties than the non-PEGylated particles for *in vivo* applications. Such system would provide the possibility of temporary PEGylation which helps overcome problems associated with PEG such as low endosomal escape.

To our knowledge, although post-insertion has been studied in lipidic nanoparticles, there have been no reports of post-insertion of PEG-lipids to siRNA polyplexes so far. Our results revealed that such phenomenon can occur but the exact mechanism remains to be investigated further. Polymers with different hydrophobicity of the backbone can be compared in the assays presented in this study to test the impact of backbone hydrophobicity on micelle-polyplex interactions. In addition, comparative silencing studies with non-PEGylated polyplexes and permanently PEGylated polyplexes can be performed to confirm whether temporary PEGylation leads to better endosomal escape properties in practise.

5. Acknowledgements

This research was financially supported by the Dutch Top Institute Pharma project T3-301.

6. Supplementary Material

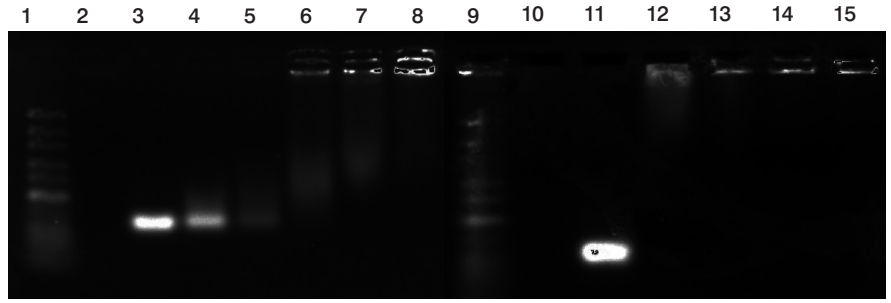

Figure 1 Gel electrophoresis image of pHPMA-MPPM-siRNA polyplex. Lanes 1, 9: marker; lanes 2, 10: pHPMA-MPPM alone; lanes 3, 11: free siRNA; lanes 4-8 and 12-15: pHPMA-MPPM-siRNA polyplex at N/P:1, 2, 3, 5, 7, 10, 14, 20, 30, respectively. siRNA concentration: 60 pmol/slot.

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4

Dual action lipoplexes for delivery of anti-angiogenic siRNA

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Submitted for publication

Abstract

Angiogenesis is one of the hallmarks of cancer which renders it an attractive target for therapy of malignancies. Tumor growth suppression can be achieved by inhibiting angiogenesis since it would deprive tumor cells of oxygen and vital nutrients. Activation of endothelial cells of tumor vasculature is the first step in angiogenesis which is mediated by various factors. One of the major triggers in this process is vascular endothelial growth factor (VEGF) which binds to VEGF receptors on endothelial cells of tumor vessels. This induces a series of signaling cascades leading to activation of cellular processes involved in angiogenesis and therefore down-regulation of VEGF receptor-2 (VEGFR-2) expression seems a viable option to inhibit angiogenesis. In our investigations, this aim has been pursued by using siRNA interfering with the expression of VEGFR-2. Since the discovery of RNA interference (RNAi) as a gene regulation process, successful delivery of small non-coding RNA has presented itself as a major challenge. In the current study, we have characterized a galectin-1 targeted anginex-coupled lipoplex (Angiplex) containing siRNA against the gene of VEGFR-2 as a dual action angiostatic therapeutic. Angiplex particles had a size of approximately 120 nm with a net negative charge and were stable *in vitro*. These particles were internalized in a specific manner by HUVECs compared to a non-targeted lipoplex system and their uptake was higher than Lipofectamine 2000. Gene silencing efficiency of Angiplex was shown to be 61%.

1. Introduction

In cancer, growth of tumors is highly dependent on sufficient distribution of oxygen and nutrients to tumor cells, which requires abundant vascular supply. When supply is limited, tumor cells can induce angiogenesis, which is the formation of new blood vessels from pre-existing vasculature, to restore [1], [2]. Therefore, this process is a crucial factor in cancer pathology and metastasis. Angiogenesis is controlled by the balance between positive and negative regulators [1]. This balance favors angiogenesis during tumor growth [3]. Hypoxia and ischemia are the main triggers of the angiogenic response causing the activation of transcription factors (such as hypoxia inducible factor) and release of growth factors. VEGF is a growth factor which plays an essential role in normal and pathological angiogenesis [4].

Endothelial cells are crucial for angiogenesis and VEGF has several biological effects on the endothelium. Firstly, it induces proliferation of endothelial cells through the activation of mitogen-activated protein kinases (MAPK) [5]. Secondly, it promotes migration and vascular leakage which are important steps in angiogenesis [6]. Thirdly, VEGF has an impact on the differentiation of hemangioblasts to endothelial cells [7]. The main binding site of VEGF on endothelial cells is FLK-1/KDR (VEGFR-2) tyrosine kinase which has high affinity for this growth factor. Interaction of VEGF with VEGFR-2 leads to VEGFR-2 dimerization and autophosphorylation resulting in the activation of certain signaling cascades which induce cellular processes involved in angiogenesis such as proliferation and migration [8]. Therefore, intervention in the VEGF signaling axis by targeting VEGF or its receptors seems an attractive therapeutic strategy as it has already been demonstrated by Hurwitz *et al.* In their study, it was revealed that in patients with metastatic colorectal cancer, combination of chemotherapy and a monoclonal antibody against VEGF (bevacizumab) improves survival remarkably [9]. In another study, DC101, an anti-VEGF monoclonal antibody, was shown to have an inhibitory effect on tumor growth by preventing new vessel formation [10].

Silencing of the expression of VEGFR-2 is an alternative approach and can be accomplished by introducing a small interfering RNA (siRNA) against VEGFR-2 mRNA into the endothelial cells of tumor vasculature. Since naked siRNA molecules are rapidly cleared and easily degraded by nucleases in blood, target localization is generally poor. Moreover, siRNA cannot be readily taken up by cells because of their relatively large size and negative charge.

Direct delivery of naked siRNA into target organs has been achieved by electroporation but the invasiveness of this method and the dependency on electrode proximity limits its applicability in humans. Gymnotic delivery has been suggested to be efficacious but this approach requires persistent and high siRNA concentrations in order to result in silencing.

Alternatively, systemic delivery of siRNA by carrier systems improves stability in blood, provides the possibility of active targeting and enhances cellular uptake. However, such systems also have specific advantages and disadvantages. Viral and non-viral carrier systems have been explored for nucleic acid delivery. Viral vectors lead to high transduction efficiencies but they have raised concerns about immunogenicity and oncogenicity when used in humans and their production is difficult. Non-viral systems, are regarded as safer and easier to produce in large scale. However, these systems are generally cationic, which upon injection can cause serious side effects.

A solution to the problems related to cationic particles would be to deliver nucleic acids by anionic particles. These complexes have been shown to cause low toxicity *in vitro* compared to cationic systems while maintaining good transfection efficiency and longer circulation times *in vivo* [11], [12]. Koldehoff *et al.*, observed low hemato-toxicity when anionic lipoplexes were administered intravenously in a patient with chronic myeloid leukaemia (CML) [13]. siRNA against bcr-abl oncogene was successfully delivered and led to an increase in apoptosis of CML cells. In another study, Zimmermann *et al.* also reported more than 90% silencing efficiency by siRNA encapsulated in stable nucleic acid lipid particles in non-human primates without toxicity [14]. The low nonspecific binding of these anionic lipoplexes in the blood compartment, prevents aggregation and allows active targeting of these particles.

In the present study, active targeting of anionic lipoplexes was explored by functionalizing the particles using a 33-mer angiostatic peptide called anginex [15]. This peptide has been reported to specifically target the tumor vasculature [16] and to use galectin-1 as its cellular receptor [17]. Galectin-1 is a lactoside-binding lectin which plays an important role in cancer. Firstly, it promotes tumor cell-endothelial cell adhesion [18], [19] and secondly, by inhibiting T-cell activation it helps tumors to escape the immune system [20]. By including anginex in the formulation of lipoplexes, on the one hand active targeting to tumor endothelial cells of the siRNA containing lipoplexes can be achieved and on the other hand, inhibition of galectin-1 may interfere directly with angiogenesis and immune recognition.

This paper describes preparation, characterization and investigation of *in vitro* efficacy of Angiplex for intracellular delivery of siRNA to HUVECs. Stability, cytotoxicity and internalization of Angiplex in HUVECs have been studied and gene knock-down efficiency has been evaluated after transfection of HUVECs with Angiplex containing siRNA against VEGFR-2.

2. Materials and methods

2.1. Materials

siRNA against human VEGFR-2 (siVEGFR-2) and negative control scrambled siRNA (NC siRNA) were purchased from Eurogentec Nederland B.V. (Maastricht, the Netherlands) with the following sequences:
siVEGFR-2: sense strand: 5´-GGA-AAU-CUC-UUG-CAA-GCU-AUU-3´, anti-sense strand: 5´-UAG-CUU-GCU-AGA-GAU-UUC-CUU-3´; NC siRNA: sense strand: 5´-CAU-CGU-CGA-UCG-UAG-CGC-AUU-3´, anti-sense strand: 5´-UGC-GCU-ACG-AUC-GAC-GAU-GUU-3´. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene-glycol)] with PEG Mw of 2000 g.mol-1 was purchased from Lipoid (Ludwigshafen, Germany). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-Mal) were supplied by Avanti® Polar Lipids Inc. (Alabaster, USA). Cholesteryl hemisuccinate (CHEMS) and protamine sulphate salt were obtained from Sigma- Aldrich (St. Louise, USA). Protected N-terminal SATA-modified peptides were synthesized by ChinaPeptides Co., Ltd. (Shanghai, China) with the following sequences:

Anginex peptide: SATA-ANIKLSVQMKLFKRHLKWKIIVKLNDGRELSLD
βpeptide-28 (βpep-28): SATA-SIQDLNVSMKLFRKQAKWKVIVKLNDGRELSLD
Aspecific peptide cyclic (Arg-Ala-Asp) was purchased from JPT (Berlin, Germany). Quant-iT™ RiboGreen® RNA Reagent and Lipofectamine 2000 were obtained from Invitrogen (Breda, the Netherlands) and Allstars Neg Control Alexa Fluor 488 siRNA (AF488 NC siRNA) was purchased from Qiagen (Venlo, the Netherlands). CytoTox-One Homogeneous membrane integrity assay was obtained from Promega Corporation (Madison, USA). All the other reagents were of analytical grade.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Verviers, Belgium) and cultured in endothelial cell growth medium-2 (EBM-2) supplemented with 2% FBS, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, and 0.1% heparin which were provided by Lonza (Verviers, Belgium). HUVECs were maintained in a humidified CO_2-incubator at 37°C and mycoplasma tests were performed regularly to ensure the absence of mycoplasma in cultures. Cells were used between passages 3-7.

2.3. Preparation and physicochemical characterization of Angiplex

Angiplex was prepared as illustrated in Figure 1. Briefly, siRNA was first complexed with protamine in 20 mM Hepes buffer containing 5% glucose at pH 7.4 (HBG) at siRNA to protamine ratio of 1:1.2 (w/w). Then DOPE, CHEMS, DSPE-PEG2000, DSPE-PEG2000-Mal were dissolved in chloroform/ethanol (3:2 v/v) in a round-bottom flask

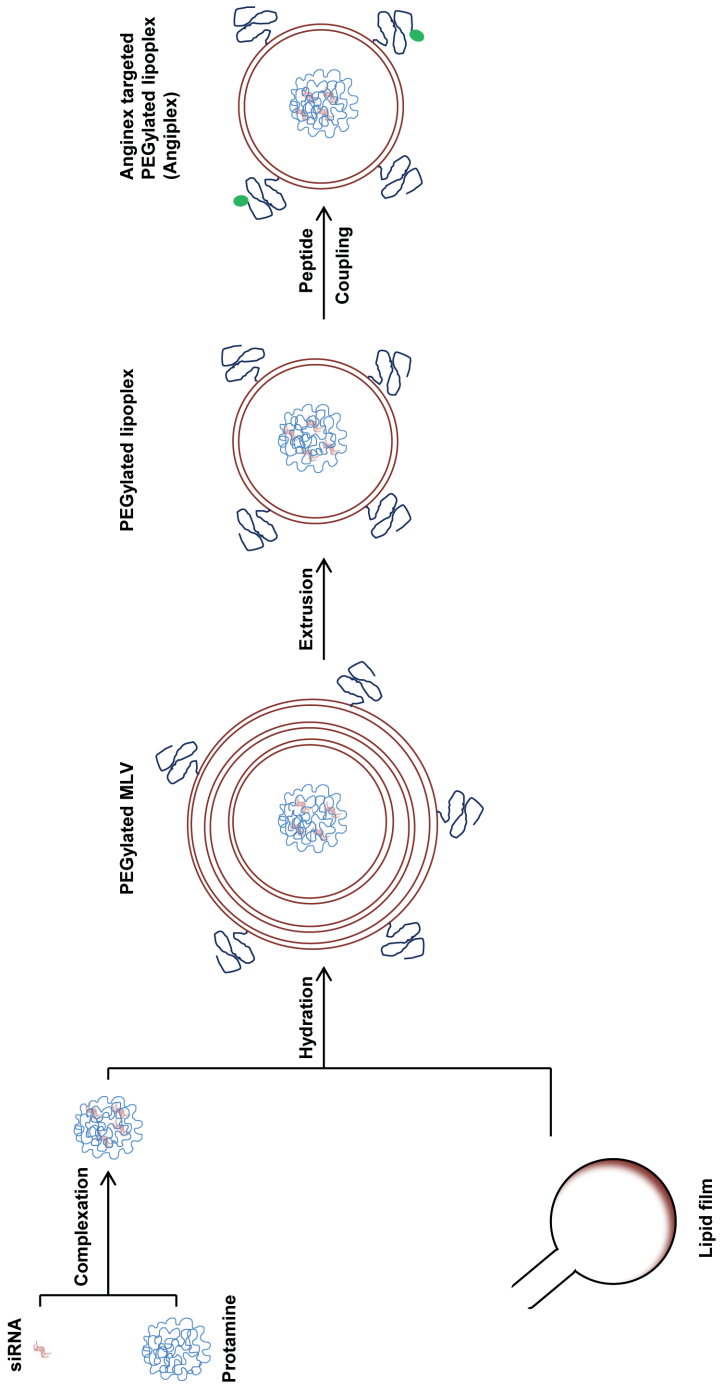

Figure 1 Schematic representation of preparation of anginex targeted lipoplexes (Angiplex). MLV: Multilamellar Vesicles.

at molar ratios of 6:4:0.3:0.3, respectively. A lipid film was prepared by solvent evaporation method and the film was hydrated by siRNA-protamine complexes. Total lipid concentration was 10 mM and siRNA concentration was 1 μM. Lipoplexes were extruded repeatedly through polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) with a final pore size of 100 nm by a Lipex™ Extruder (Northern Lipids, Burnaby, BC, Canada).

A solution of 0.05 M Hepes, 0.05 M hydroxylamine, and 0.03 mM EDTA at pH 7.0 was added to SATA-anginex at volume ratio of 1:10 and was incubated for 45 minutes at room temperature to deprotect the SATA groups. Deprotected peptides were then added to lipoplexes at a concentration of 10 μg peptide per 1 μmol phospholipid and left at 4°C overnight. Uncoupled peptides were separated from the peptide-modified lipoplexes by ultracentrifugation for 60 minutes at 200,000 g at 4°C [21]. 10-fold molar excess of maleimide groups of DSPE-PEG2000-Mal to SATA groups of SATA-anginex was calculated to ensure complete coupling. Coupling of the negative control peptide (SATA-bpep-28) was performed in the same manner.

Hydrodynamic diameter of lipoplexes was measured at 25°C by dynamic light scattering on a Malvern 4700 system using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern Instruments Ltd., Worcestershire, UK). 90° angle was used for all measurements and viscosity and refractive index of water at 25°C were applied. System calibration was done by standard polystyrene beads with a diameter of 200 nm (Thermoscientific, DE, USA). Size distribution of lipoplexes was presented by PDI (polydispersity index) ranging from 0 indicating a monodisperse sample to 1 for a polydisperse sample. ζ- potential was determined at 25°C by Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, UK). Calibration of the apparatus was done by Zeta Potential Transfer Standard (Malvern Instruments Ltd., Worcestershire, UK) with a known ζ- potential.

2.4. Transmission Electron Microscopy (TEM)

Morphology of Angiplex was visualized by TEM with a Philips Tecnai 10 equipped with a Biotwin lens and a LaB6 filament (100 kV acceleration voltage). 10 μL of lipoplexes was pipetted on a piece of Parafilm and a glow discharged copper grid (200 mesh with a carbon coated thin film) was placed on top of it. After 2 minutes, the excess liquid was removed by filter paper and the grid was negatively stained with 2% uranyl acetate (Merck) and left for 5 minutes before capturing the images with a SIS Megaview II CCD-camera.

2.5. Agarose gel electrophoresis

Lipoplexes were prepared at siRNA concentration of 1 μM (20 pmol per gel slot) in HBG and mixed with Triton X-100 (5% w/v) and dextran sulfate sodium (DSS; 1% w/v).

They were then incubated for 20 minutes at room temperature and applied to the slots of 4% (w/v) agarose gels. Tris-Acetate-EDTA was used as running buffer and electrophoresis was performed at 100 V for 45 minutes. Gelred™ (Phenix Research Products, NC, USA) was used to stain RNA after electrophoresis according to the manufacturer's protocol and images were made with UV transilluminator (ImaGo compact imaging system (B&L Systems), the Netherlands).

2.6. LDH cytotoxicity assay

HUVECs were seeded at 4,000 cells/well in a 96-well plate 24 h prior to transfection. Then, the cells were transfected with formulations containing 38 nM RNA in EGM-2 complete medium. After 4 h of incubation at 37°C in CO_2-incubator, LDH assay was performed with CytoTox-ONE™ kit (Promega, Madison, WI, USA) as described by the manufacturer.

2.7. Cell association and internalization

For cell association studies, HUVECs were seeded on ice in 96 well-plates at a concentration of 50,000 cells/well. Treatments containing 0.5 μM RNA were added immediately and the plates were left at 4°C for 1 h. Then, cell pellets were obtained by centrifugation of plates at 250 g for 5 minutes at 4°C. Pellets were washed 3 times with 0.3 % BSA in PBS and fixed in 10% formalin.

For cell internalization experiments, HUVECs were seeded in 24 well-plates at concentration of 50,000 cells/well 24 h prior to transfection. Transfections were performed with formulations containing 0.2 μM RNA in EGM-2 complete medium. Cells were incubated for 4 h at 37°C and then medium was changed with fresh EGM-2 complete medium. After another 44 h incubation time at 37°C, cells were washed with acid wash solution (0.2 M glycine and 0.15 M NaCl at pH 3), detached and fixed in 10% formalin.

Flow cytometry analysis was performed by Becton & Dickenson FACSCalibur flow cytometer to determine mean fluorescence intensity per cell. 10,000 events were recorded per sample and data analysis was done by FACSDiva™ software (Becton & Dickenson, Mountain View, CA, USA). GraphPad Prism 4 software was used for statistical analysis and one-way ANOVA with a Bonferroni post-test was used to calculate statistical significance.

2.8. Confocal microscopy

HUVECs were seeded in 16-well chamberslides (Thermo Fisher Scientific, MA, USA) at concentration of 10,000 cells/well 24 h before transfection. Thereafter, medium was removed and cells were washed with 5% BSA in PBS on ice. Lipoplexes containing DiI (0.1 mol % of total lipid) and AF488 NC siRNA were added to cells at RNA concentration of 152 nM. Chamberslides were left for 1 h at 4°C in the dark after which

they were washed two times with PBS and incubated for 1 h at 37°C in CO_2-incubator. Cells were then fixed in 10% formalin and incubated for 5 minutes at room temperature with DAPI for nuclei staining. Slides were mounted by FluorSave (Calbiochem, San Diego, CA, USA). A Zeiss Axiovert 200 M confocal microscope (Carl Zeiss Microscopy GmbH, Germany) was used for obtaining the images. The microscope had a ×63 oil immersion objective (NA 1.2) plus ×2 digital zoom. An air-cooled argon ion laser (LASOS, RMC 7812Z, 488 nm) for FITC and a HeNE (LASOS, SAN 7450A, 543 nm) laser for DyLight were used for excitation.

2.9. Competition assay

HUVECs were seeded in 24 well-plates at concentration of 50,000 cells/well 24 h before transfection. On the day of transfection, cells were washed once with PBS and free anginex was added immediately followed by addition of treatments. Separate plates were used for each concentration of free anginex (0, 10, or 30 μg/ml) and the experiment was done in triplicates. Treatments included Angiplex, bpep-28-lipoplexes (bpep-28-L), and bare lipoplexes (Bare-L) all containing AF488 NC siRNA. Plates were then incubated for 3 h at 37°C in CO_2-incubator. After the incubation period, cells were washed with PBS, acid wash solution (0.2 M glycine and 0.15 M NaCl at pH 3) and another time with PBS. Then they were detached and fixed in 10% formalin. Flow cytometry analysis was performed as described in section 2.7.

2.10. Western Blotting

HUVECs were seeded in 6 well-plates at concentration of 50,000 cells/well 24 h prior to transfection. Cells were transfected 4 times with 1 h incubation-intervals with formulations containing 38 nM RNA (siVEGFR-2 or NC siRNA), and finally incubated for 48 h at 37°C. Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer supplemented with EDTA and protease inhibitors (Thermoscientific, DE, USA) and protein concentration was determined by MicroBCA Assay (Thermoscientific, DE, USA). Samples containing 10 μg reduced protein were loaded on NuPAGE® 4-12% Bis-Tris polyacrylamide gel (Life Technologies, NY, USA) and electrophoresis was performed at 150 V for 45 minutes. Proteins were transferred from the gel to nitrocellulose membrane using the iBlot® Dry Blotting system (Life Technologies, NY, USA). Membranes were blocked with 5% BSA in TBS-T (Tris-buffered saline containing 0.1% Tween20 at pH 7.4) for 1 h at room temperature. After three washing steps with TBS-T, membranes were incubated with primary antibody against b-actin (Cell Signaling Technology, OR, USA) or VEGF Receptor2 Rabbit mAb (Cell Signaling Technology, OR, USA) for 2 h at room temperature. Then, the blots were washed 3 times with TBS-T and were incubated with Peroxidase Conjugated Stabilized Goat Anti-Rabbit IgG (H+L) (Thermoscientific, DE, USA) for 2 h at room temperature. All primary and secondary antibodies were diluted 1:1000 (v/v) in 5% BSA in TBS-T.

Visualization of proteins was performed by SuperSignal West Femto Chemiluminescent Substrate (Thermoscientific, DE, USA) as indicated by the manufacturer and images were made by Bio-Rad CHEMDOC XRS (Veenendaal, the Netherlands). Bands were quantified by ImageJ 1.45s (NIH, USA).

3. Results and discussion

3.1. Preparation and characterization of Angiplex

Angiplex was prepared in two steps. First, siRNA was complexed with protamine. Pre-complexation of siRNA with polycationic molecules is a common strategy to increase the encapsulation efficiency of these small RNA molecules inside neutral or negatively charged vesicles [22], [23], [24], [25], [26]. Second, siRNA-protamine complexes were coated with lipid bilayers by the lipid film hydration method. In the composition of the lipid bilayers DOPE (a conelike fusogenic lipid) was used which causes transition of the lipid bilayer from lamellar phase (L_α) to hexagonal phase (H_{II}) in acidic pH by creating negative curvature [27], [28]. This property is beneficial in endosomal escape which is usually the limiting step in nucleic acid delivery. In presence of DOPE, the lipid bilayer can fuse to the endosomal membrane when the pH drops and therefore the content is released into the cytosol. Another important phospholipid in the formulation was DSPE-PEG2000 which, due to the PEG-chains, prevents aggregation. In addition, it may provide stealth properties to the particles which is important for circulation time. It has been shown in the literature that PEG decreases the pH-dependent activity of DOPE as a result of stabilizing the particles [29], [30] but since PEG itself possesses fusogenic properties, it has a net contribution to the efficacy of DOPE liposomes [31]. In the composition of the lipoplexes, DSPE-PEG2000-Mal has been used to attach the targeting peptide anginex or control peptide bpep-28 to the distal ends of PEG-chains.

As indicated in Table 1, the resulting peptide-coupled lipoplexes were relatively monodisperse with an average size of ~120 nm and a net negative charge of -15 mV. Coupling of peptides did not change size and charge of the particles significantly. These particles have attractive size for nanomedicine applications and their PEGylation should prevent opsonization by plasma proteins and improve their circulation half-life. The net negative charge of the final preparations creates an advantage over cationic liposomes. Positively-charged particles interact more with plasma proteins than their negatively-charged counterparts which leads to their rapid clearance and undesirable pharmacokinetics [32], [33]. Also, they result in activation of immune response and thereby toxicity [34], [35], [36], [37].

Encapsulation efficiency of siRNA in Angiplex was determined by quantifying the amount of siRNA present in the formulation using RiboGreen®. For this, particles

were first disrupted with 5% (w/v) of Triton X-100 and 1% (w/v) of DSS. Then the percentage of siRNA entrapped in the lipoplexes was calculated by subtracting the amount detected for non-treated lipoplexes from the amount detected for disrupted lipoplexes. Based on the RiboGreen® quantification it was calculated that 51% of the initial amount of siRNA was entrapped inside the lipoplexes.

As control formulation, lipoplexes with siRNA equipped with bpep28 (bpep-28-L), empty Angiplex, and bare lipoplexes (Bare-L) were prepared in the same way as described above and there was no considerable difference between size and charge of these formulations and that of Angiplex (Table 1).

Morphology of Angiplex was investigated by TEM (Figure 2). Vesicular structures and tubular structures were observed. Based on the method of particle preparation it is likely that the vesicular structures represent typical liposomal bilayers encompassing an aqueous core.

Table 1 Mean size and mean zeta potential of lipoplexes (n=3)

Sample Name	Size (nm) ± SD	PDI	Zeta Potential (mV) ± SD
siVEGFR-2 protamine lipoplex (Bare-L)	104 ± 7	0.1	-13 ± 1
siVEGFR-2 protamine Ax lipoplex (Angiplex)	124 ± 3	0.2	-15 ± 1
siVEGFR-2 protamine bpep-28 lipoplex (bpep-28-L)	124 ± 1	0.1	-15 ± 1
Empty Ax lipoplex	106 ± 1	0.1	-13 ± 1

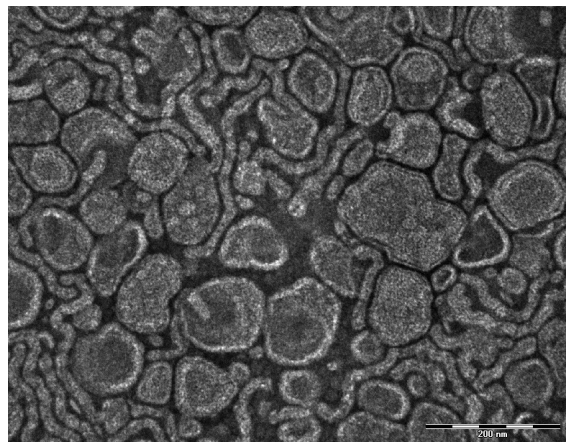

Figure 2 TEM image of Angiplex. Scale bar shows 200 nm.

3.2. Angiplex is stable and maintains integrity of siRNA

To investigate complexation stability of Angiplex, gel retardation experiments were done in the presence or absence of 5% (w/v) of Triton X-100 and 1% (w/v) of DSS. Angiplex released siRNA only after the formulations were treated with 5% (w/v) of Triton X-100 and 1% (w/v) of DSS (Figure 3, column 5). Maintaining nucleic acid integrity is essential for an intravenously injected carrier because in stably-complexed siRNA carriers less displacement of siRNA in the formulation with negatively charged serum components occurs [38].

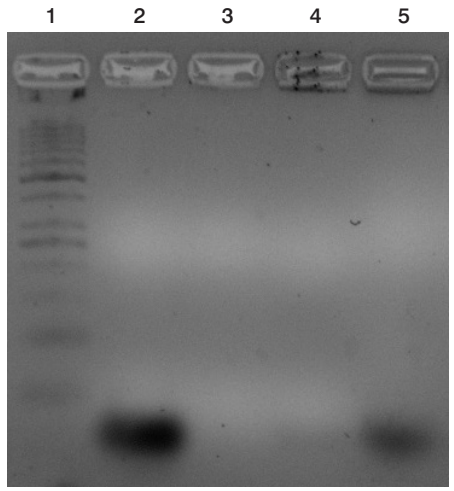

Figure 3 Gel electrophoresis of Angiplex before and after treatment with Triton X-100 and DSS to evaluate complexation stability (1: Marker, 2: Naked siVEGFR-2, 3: Empty Angiplex, 4: siVEGFR-2-Angiplex, 5: siVEGFR-2-Angiplex+Triton X-100 5%+DSS 1%).

3.3. Cellular toxicity is significantly lower after treatment with Angiplex than with cationic liposomes

In order to evaluate cytotoxicity, HUVECs were transfected with Angiplex, positively-charged DOTAP liposomes (DOTAP/Cholesterol at a molar ratio of 1:1) and a commercially available transfecting agent, Lipofectamine 2000. All formulations contained 38 nM NC siRNA. 4 h after transfection, release of LDH was measured and reported relative to that of non-treated cells as measure of cytotoxicity. Release of LDH was 13 times lower in cells treated with Angiplex compared to DOTAP liposomes and 5 times lower than Lipofectamine 2000-treated cells (Figure 4). This effect could

be related to PEGylation and the negative charge at the surface of Angiplex which cause less adsorption to cell-membranes with concomitant reduction of membrane integrity disruption.

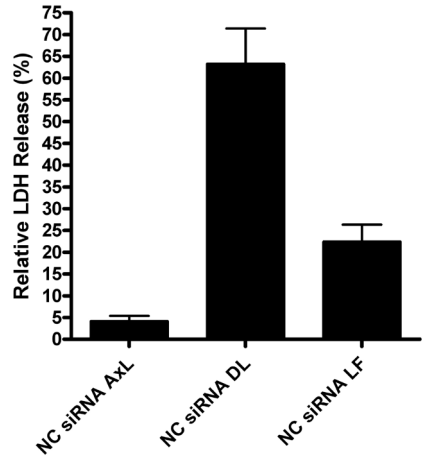

Figure 4 Toxicity of Angiplex (AxL) compared to DOTAP liposomes (DL) and Lipofectamine 2000 (LF) in HUVECs 4 h after addition of formulations. Release of cytosolic lactate dehydrogenase (LDH) indicates toxicity (n=5).

3.4. Angiplex binds to HUVECs and is internalized

Cell association of Angiplex to galectin-1 was investigated in HUVECs. To detect only the particles bound to the cell surface and not internalized, we performed our studies at low temperatures. Fluorescently-labeled siRNA (AF488 NC siRNA) was incorporated in the particles to allow detection of cell association by flow cytometry. Results were expressed as mean fluorescence intensity (MFI) relative to that of non-treated samples. Cell association of Angiplex was 10-fold higher than that of Lipofectamine 2000 which could be attributed to multimeric display and high affinity of anginex to galectin-1 on HUVECs (Figure 5A) [17]. The association of Lipofectamine 2000 particles is likely not based on receptor interaction but rather on charge-based interactions with negatively charged surface molecules. A possible factor that could contribute to the difference is different quenching of fluorescent siRNA in Angiplex and in Lipofectamine 2000. Binding of bpep-28-L and Bare-L were below detection limit, likely because of lack of specific interaction. These last two controls show that the surface is indeed little interactive.

In the internalization studies, in order to remove the surface-bound particles the cells were washed with acid wash solution (0.2 M glycine and 0.15 M NaCl at pH 3) prior to fixation and then flow cytometry was performed. Angiplex was internalized by HUVECs 1.5-fold more than Lipofectamine 2000 and fluorescence intensities were noticeably higher compared to bpep-28-L and Bare-L (Figure 5B). This observation could be related to the fact that Angiplex is taken up specifically via ligand-receptor as well as non-specific interactions whereas Lipofectamine 2000 complexes are internalized via charge interactions in a non-specific way. Uptake of other formulations were hypothesized to be mainly through non-specific endocytosis.

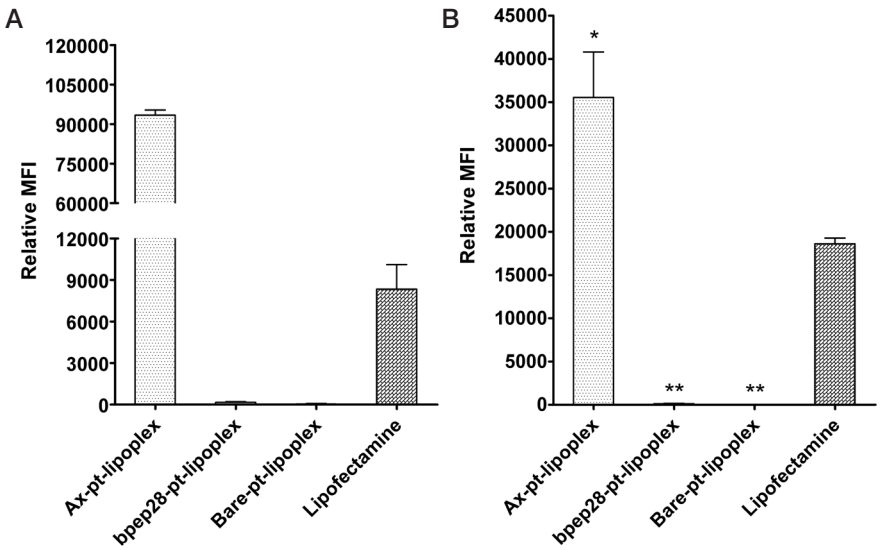

Figure 5 **(A)** Cell binding of Angiplex in comparison with bpep-28-L and lipofectamine 2000 in HUVECs 1 h after transfection. All treatments contained 0.5 μM of AF488 NC siRNA (n=3). **(B)** Cell uptake of Angiplex in comparison with bpep-28-L and Lipofectamine 2000 in HUVECs 48 h after transfection at AF488 NC siRNA concentration of 0.2 μM (n=3). $p<0.05$ is denoted by * and $p<0.001$ by **.

Confocal microscopy images taken after transfection of HUVECs with lipoplexes confirmed the above findings. Cells were first incubated for 1 h at 4°C and then washed with PBS to remove non-bound particles. Then, they were incubated for 1 h at 37°C to provide enough time for the attached particles to be internalized. A

punctuate pattern of green fluorescence from AF488 dye was seen in the cytosol in case of samples treated with Angiplex which was absent in cells treated with bpep-28-L and Bare-L (Figure 6). These dots which indicated the presence of siRNA could point to the entrapped particles in the endosomes. The diffused red fluorescence observed in the cytosol of the cells could be due to DiI dye which is incorporated in the liposomes as well as autofluorescence since it is also observed in the non-treated samples. The red color was present in all samples although it was the strongest in Angiplex-treated cells.

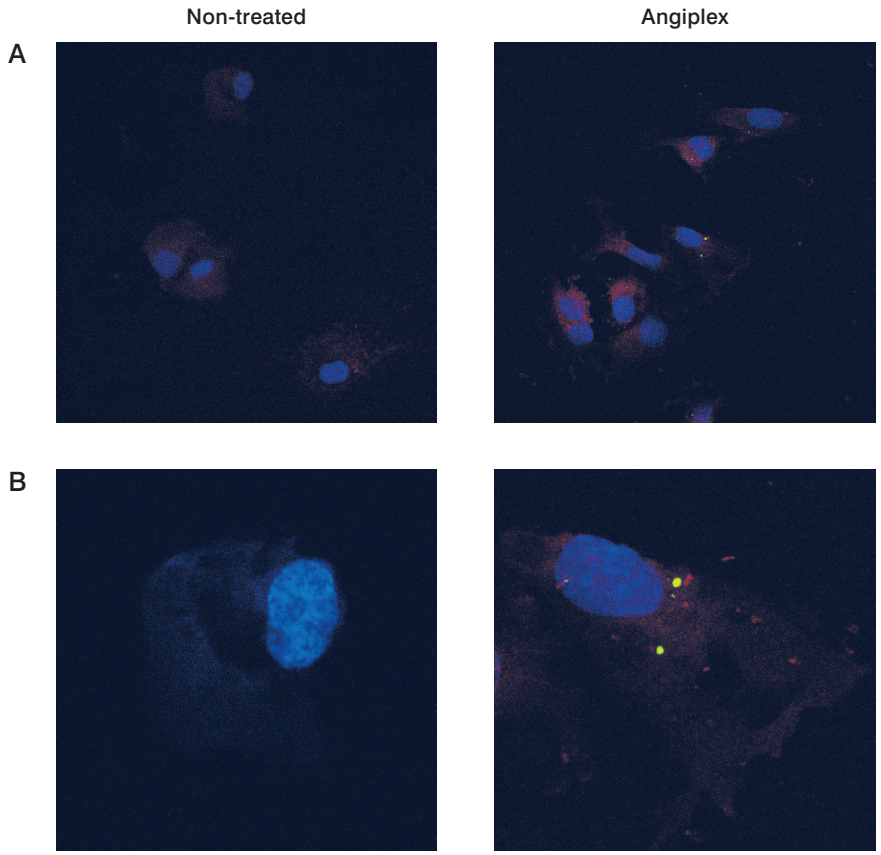

Figure 6 Confocal microscopy images of HUVECs incubated with treatments for 1 h at 4°C, washed with PBS and then incubated for 1 h at 37°C. Panels **(B)** and **(D)** show the same cells from panels **(A)** and **(C)**, respectively, with a higher zoom. Different colors of staining reflect the following: blue for cell nuclei, red for liposomes, and green for siRNA.

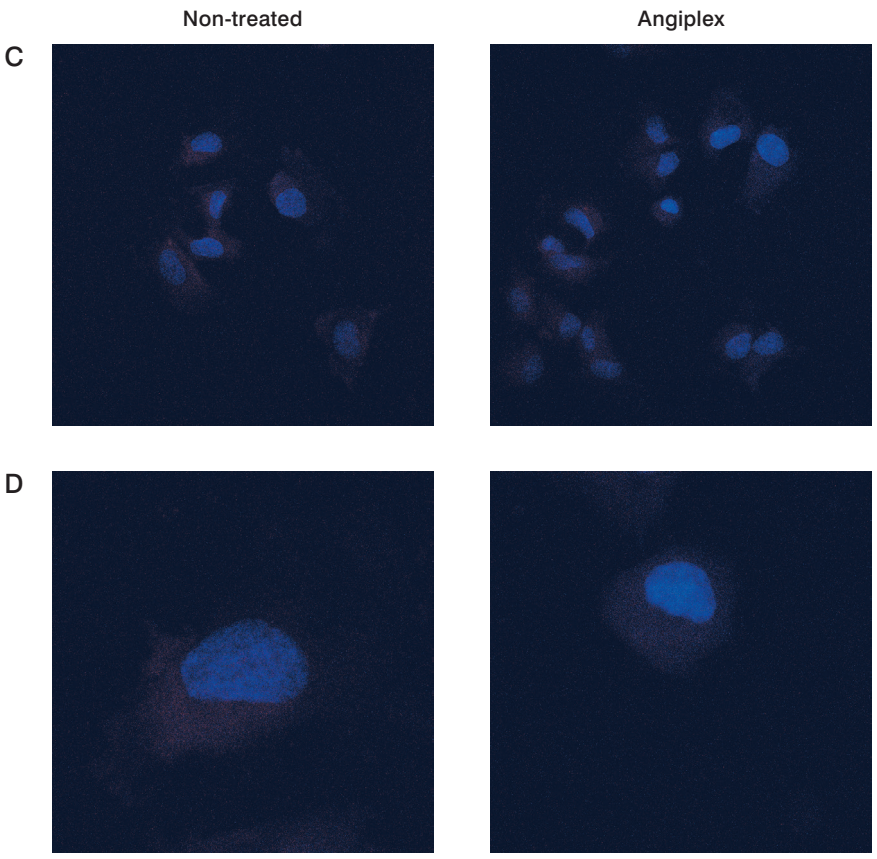

Figure 6 Continued.

3.5. Uptake of Angiplex in HUVECs is peptide-dependent

In order to assess whether the uptake of lipoplexes was through specific anginex-galectin-1 interaction, competition assays were performed. HUVECs were incubated with particles competing with free anginex for 3 h at 37°C. The amount of free anginex added to the treated cells was at the following molar ratios compared to coupled anginex: 0:1, 1:1, 3:1 corresponding to free anginex concentrations of 0, 10, and 30 µg/ml. After removing non-bound particles, flow cytometry measurements were done. Fluorescence intensity reflected the level of cell internalization. A drop in fluorescence signal was observed for Angiplex when the concentration of free anginex increased from 10 to 30 µg/ml while this effect was absent for bpep-28-L and Bare-L (Figure 7). There was no significant difference of fluorescence at free anginex

concentrations of 0 and 10 µg/ml. It was hypothesized that a minimum concentration of free anginex is needed to block enough galectin-1 receptors and result in a measurable effect. These results demonstrate specific uptake of Angiplex through anginex.

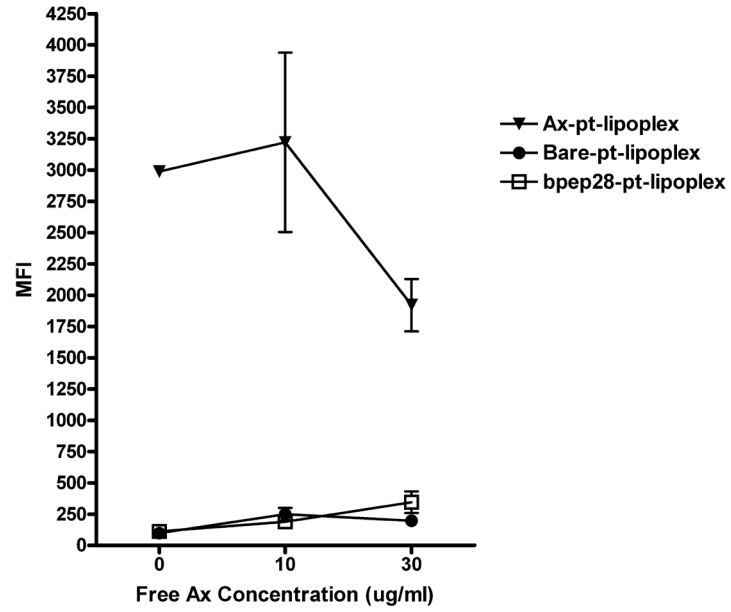

Figure 7 Competition of Angiplex with free anginex in comparison with bpep-28-L and Bare-L at free anginex concentrations of 0, 10, and 30 µg/ml. MFI represents mean fluorescence intensity (n=3).

3.6. Angiplex reduces expression of VEGFR-2 by delivering siRNA complementary to mRNA of VEGFR-2

Biological activity of delivered siVEGFR-2 was evaluated 48 h after treatment of HUVECs with Angiplex. Transfections were done multiple times with 1 h time intervals to mimic the constant clearance of particles in the blood stream *in vivo*. bpep-28 was used as a non-targeting peptide mimicking anginex since it has the same length of 33 amino acids, 22 of which are identical to anginex. Moreover, an aspecific negative control peptide (asp-NCpep) with no similarities to anginex was used to assess any differences that might occur due to resemblance of bpep-28 to anginex. Down-regu-

lation of VEGFR-2 was 61% when HUVECs were treated with Angiplex (Figure 8). Angiplex showed a dual action by both RNAi and the binding of galectin-1 to neuropilin-1 which can trigger the down-regulation of VEGFR-2-mediated signaling pathway [39]. Another observation was that treatments containing negative control peptides resulted in similar silencing efficiency to Angiplex. As this effect was not observed in the uptake study (section 3.5) it could be concluded that the different set-up of the gene knock-down experiment (maintaining the formulations with the cells for 48 h) versus the internalization study (4 h incubation of the formulations with the cells) has led to remarkable non-specific uptake.

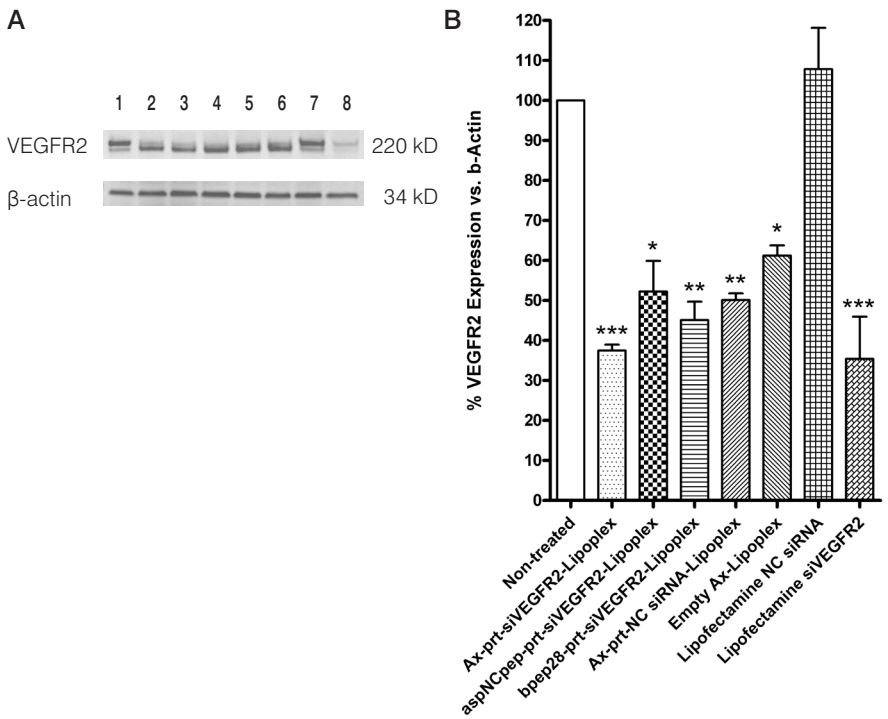

Figure 8 **(A)** Western blot of VEGFR-2 48 h after transfection of HUVECs with lipoplexes containing siVEGFR-2, NC siRNA or empty lipoplexes at siRNA concentration of 0.2 μM (1: Non-treated, 2: Angiplex-siVEGFR-2, 3: *asp-NCpep*-L-siVEGFR-2, 4: *bpep-28*-L- siVEGFR-2, 5: Angiplex-NC siRNA, 6: Empty Angiplex, 7: Lipofectamine 2000-NC siRNA, 8: Lipofectamine 2000-siVEGFR-2). b-actin was used as loading control. **(B)** Quantification of silencing efficiency (n=3). $p<0.05$ is denoted by *, $p<0.01$ by **, and $p<0.001$ by ***.

Compelling evidence has indicated that simultaneous targeting of different pathways in tumor cells offers more efficiency benefits compared to a single-targeted approach because it can potentially circumvent drug resistance [40], [41], [42], [43]. For example, anti-prostate-specific membrane antigen (PSMA) RNA aptamer conjugated to gelonin toxin, improved toxicity in prostate tumor cells [44]. In another study, siRNA against signal transducer and activator of transcription 3 (STAT3) was delivered by a B-cell-activating factor (BAFF)-receptor aptamer targeting B-cells. A remarkable combined effect was observed by inhibition of B-cell proliferation via interaction of the aptamer with BAFF-receptor and decrease of STAT3 mRNA levels through RNAi in B-cells [45]. EGa1 nanobody-coupled liposomes loaded with the AG538 (inhibitor of IGF-1R) have been shown to downregulate EGFR expression with a higher efficacy than either empty EGa1-coupled liposomes or non-targeted AG538 liposomes. This effect has been attributed to the simultaneous inhibition of EGFR activation by EGa1 nanobody and IGF-1R inhibition by AG538 [46]. Our findings have revealed that dual action of Angiplex has caused a double effect on a cellular process through combining a ligand-receptor interaction event and manipulating cellular pathways at different levels. Hsieh *et al.* have reported that binding of galectin-1 to neuropilin-1 down-regulates the VEGFR-2 pathway [39]. Moreover, it has been reported that anginex liposomes *per se* have anti-angiogenic capabilities because of anginex [21]. The above observations could explain how empty angiplex particles in our study led to silencing of VEGFR-2 gene.

4. Conclusion

In this study, anginex lipoplexes (Angiplex) encapsulating siRNA were prepared with an average size of 120 nm and a net negative charge. These complexes showed low cell toxicity and their high cell internalization resulted in up to 61% gene knock-down. Surprisingly, the lipoplex we developed led to an intrinsic down-regulation of VEGFR-2 irrespective of RNA-payload or targeting ligand. We are currently exploring the underlying mechanism behind this phenomenon. Taken together, Angiplex can be considered as a promising dual action candidate for anti-angiogenic therapy through siRNA delivery and anginex activity.

5. Acknowledgements

This research was financially supported by and performed within the framework of the Dutch Top Institute Pharma project T3-301. We thank Raimond Heukers for assistance with confocal microscopy experiments and Joep van den Dikkenberg for TEM experiments.

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A comparison of hemocompatibility between a lipid-based and a polymer-based siRNA nanocarrier formulation

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Submitted for publication

Abstract

Since the discovery of RNAi and its therapeutic potential, carrier systems have been developed to deliver small RNAs (siRNA in particular) for modulation of gene expression at the post-transcriptional level. An important factor determining the fate and usability of these carrier systems *in vivo* is their interaction with blood components, blood cells, and the immune system. In this paper, we have studied hemocompatibility of two nanocomplexes containing siRNA in terms of activation of platelets, coagulation and complement cascade in addition to induction of platelet aggregation. The nanocomplexes studied were Angiplex which is a lipid-based system and pHPMA-MPPM polyplex which is a formulation based on cationic polymers. Both systems have recently been shown to cause significant silencing *in vitro.* Moreover, Angiplex was studied with respect to activation of innate immune responses. Our findings indicated that pHPMA-MPPM polyplex triggered high platelet activation and aggregation although it did not stimulate the contact system of coagulation substantially. Angiplex, on the other hand, provoked insignificant activation and aggregation of platelets and activated the contact system minimally. It also did not lead to significant immune stimulation via the TLR pathway. Activation of the complement system (C system) by Angiplex was in general low but stronger than pHPMA-MPPM polyplex. Taken together, these *in vitro* assays may help in the selection of suitable carrier systems for systemic delivery of siRNA in early preclinical investigations and thus reduce the use of laboratory animals significantly.

1. Introduction

Unmodified siRNA is prone to fast degradation by nucleases in the blood after systemic administration. In addition, large size and negative charge of siRNA hampers internalization of these molecules into the cells. Therefore, delivery systems are being investigated to protect siRNA in the circulation and increase their cellular uptake. Two main categories of synthetic carriers used for siRNA delivery are polyplexes and lipoplexes where siRNA is complexed with a polymer or with lipids, respectively. We have recently observed the successful delivery of a targeted lipoplex (Angiplex) containing siRNA against VEGFR-2 to HUVECs (manuscript submitted for publication). Also, we have reported on prominent gene silencing efficiency of pHPMA-MPPM complexes with siRNA against firefly luciferase in H1299 human lung cancer cells expressing firefly luciferase [1]. Nevertheless, translating *in vitro* results to the *in vivo* situation still remains a challenge in the field as it has been shown that efficient delivery systems *in vitro* might not lead to optimal *in vivo* results [2], [3], [4]. Many factors need to come together to obtain successful transfection, such as stability in the circulation, lack of toxicity, extravasation to the target tissue, target cell entry and endosomal escape [5], [6]. In this paper, we are focusing on the initial interactions with the blood when nanocarriers enter the systemic circulation as well as the immune response to the presence of these particles. After intravenous administration, nanoparticles first come into contact with blood components (such as albumin, coagulation factors and proteins of the complement system), blood cells (for example platelets), and the cells of the immune system. Since these interactions could negatively affect their delivery to the target site or even worse, trigger systemic toxicity, it is vital to evaluate behavior of such particles in the blood before proceeding to *in vivo* studies.

Regarding platelets, in addition to being involved in inflammation and hypersensitivity, these cells play an important role in primary hemostasis, which is the blocking of hemorrhage proceeding vascular injury. Aside from trauma, platelets can become activated upon encountering foreign particles (bacteria, viruses, and nanomedicine) which could also lead to thrombotic occlusion of the lumen of vessels and subsequently tissue damage.

Secondary hemostasis is regulated by the coagulation cascade which occurs through extrinsic (or tissue factor (TF) coagulation) pathway and intrinsic (or contact activation) pathway. Studying the influence of drug carriers on activation of platelets and the contact system could assist in predicting *in vivo* fate of these nanoparticles.

Furthermore, it is critical to consider how the host immune system responds to nanomedicine in order to prevent severe immune reactions. Recognition of nanoparticles occurs through toll-like receptors (TLRs) which are predominantly expressed in dendritic cells (DCs) and macrophages. One of the main concerns

regarding siRNA delivery is the risk of TLR3 activation by these molecules since TLR3 recognizes double-stranded RNA (dsRNA) as a microorganism-associated molecular pattern (MAMP) associated with virally infected cells [7], [8]. Other TLRs are involved in recognition of different MAMPs among which we have focused on the following in this study: TLR2, 4, and 9.

Another key player in host defense is the C system, the activation of which has been reported to be a cause of low transfection efficiency of non-viral nucleic acid delivery systems [9]. C system can be activated by nanomedicine and lead to opsonization and thus rapid elimination of these particles. In this paper, we have investigated thrombogenicity, effect on coagulation, complement activation, and immunogenicity of Angiplex and pHPMA-MPPM polyplex and how physicochemical characteristics of these particles determine these interactions.

2. Materials and methods

2.1. Materials

Human siRNA against VEGFR2 (siVEGFR-2) with the following sequence: sense 5´-GGA-AAU-CUC-UUG-CAA-GCU-AUU-3´ and anti-sense 5´-UAG-CUU-GCU-AGA-GAU-UUC-CUU-3´ was purchased from Eurogentec Nederland B.V. (Maastricht, the Netherlands). Poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol (pHPMA-MPPM) 240 kDa was synthesized and purified in our group as described previously [10]. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)] with PEG Mw of 2000 g.mol-1 was obtained from Lipoid (Ludwigshafen, Germany). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(poly-ethylene glycol)-2000] (DSPE-PEG2000-Mal) were supplied by Avanti® Polar Lipids Inc. (Alabaster, USA). Cholesteryl hemisuccinate (CHEMS) and protamine sulphate salt from salmon (Grade X) were obtained from Sigma-Aldrich (St. Louise, USA). Protected N-terminal SATA-modified anginex peptide was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China) with the following sequence:
SATA-ANIKLSVQMKLFKRHLKWKIIVKLNDGRELSLD

JetPEI was obtained from Polyplus Transfection (Illkirch, France) and Lipofectamine 2000 was purchased from Invitrogen (Breda, the Netherlands). Thrombin Receptor Activator Peptide 6 (TRAP-6) and Chromogenic substrate L-2120 (H-D-Pro-Phe-Arg-pNA) were obtained from Bachem (Bubendorf, Switzerland). PE-conjugated anti P-selectin was acquired from BD Biosciences (Breda, the Netherlands). FITC-conjugated polyclonal rabbit anti human fibrinogen was purchased from DAKO (Glusdorp, Denmark). Blood was obtained from the Mini Donor Dienst (MDD) (UMC, Utrecht, the Netherlands). Kaolin (Light) was purchased from BDH Ltd. (Poole, UK). Citrated

human normal pooled plasma was prepared from the whole blood of approximately 170 healthy volunteers, according to standardised procedures [11]. 1,2-Dioleyl-sn-glycero-3-phospho-L-serine (PS), 1,2-dioleyl-sn-glycero-3-phosphocholine (PC), and 1,2-dioleyl-sn-glycero-3 phosphoethanolamine (PE) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Prostacyclin (PGI_2) (100 µg/ml) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Microvue SC5b-9 Plus ELISA kits were provided by Quidel Co. (CA, USA). All the other reagents were of analytical grade.

2.2. Cell culture

TLR reporter cell lines (HEK-Blue™-hTLR2, -hTLR3, -hTLR4, and –hTLR9 reporter cells) were cultured as instructed by the manufacturer (Invivogen, Toulouse, France).

2.3. Methods

2.3.1. Preparation and characterisation of Angiplex and pHPMA-MPPM polyplex

Angiplex was prepared from DOPE, CHEMS, DSPE-PEG2000-Mal, and DSPE-PEG2000 at molar ratios of 6:4:0.3:0.3, respectively. Lipids were dissolved in ethanol:chloroform (4:3 v/v) on a rotavapor (Buchi, Switzerland) at 40°C after which a lipid film was made by evaporation of the solvents under vacuum. Meanwhile, an siRNA-containing core was prepared from siVEGFR-2 and protamine at ratio of 1:1.2 (w/w) in 20 mM HEPES buffer containing 5% glucose at pH 7.4 (HBG). After incubating this mixture for 20 minutes at room temperature, the complex was added to the lipid film. Total lipid concentration of lipoplexes was 10 mM and siRNA concentration was 1 µM. The lipoplex suspension was extruded repeatedly through polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) by a Lipex™ Extruder (Northern Lipids, Burnaby, BC, Canada) until a monodisperse sample was obtained with an approximate size of 100 nm. Then anginex-coupling was performed by firstly removing the protective group (SATA) of the peptide with a deprotecting solution (0.05 M HEPES, 0.05 M hydroxylamine and 0.03 mM EDTA at pH 7.0) at a peptide to deprotecting solution ratio of 1:10 (v/v). The reaction was completed by incubation for 45 minutes at room temperature. Secondly, the unprotected anginex was mixed with lipoplexes and incubated over night at 4°C. Uncoupled peptide was removed by ultracentrifugation at 200,000 g for 1 h at 4°C. Anginex concentration was calculated as 10 µg per µmol phospholipid. Bare lipoplexes were prepared in the same manner excluding the peptide-coupling step.

Polyplexes consisted of siVEGFR-2 complexed with pHPMA-MPPM at N/P ratio of 7 (where N represents the moles of cationic nitrogens in the polymer and P represents the moles of phosphate groups in siRNA). siVEGFR-2 (2.1 µM) was added to the polymer in buffer containing 20 mM HEPES and 150 mM NaCl (HBS) at pH 5, vortexed for 5 seconds, and incubated for 30 minutes at room temperature.

Hydrodynamic diameter of lipoplexes and polyplexes was determined by dynamic light scattering on a Malvern 4700 system at 25°C using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern Instruments Ltd., UK). Measurement angle was 90° and viscosity and refractory index of water at 25°C were applied. The apparatus was validated by standard polystyrene beads with a size of 200 nm (Thermoscientific, DE, USA). Polydispersity index (PDI) obtained from the measurements, represents size distribution of particles which can vary between 0 and 1 where 0 indicates single sized particles and 1 a heterogeneous mixture of particles. Charge of particles was characterised by Zetasizer Nano-Z (Malvern Instruments Ltd., UK) which measures the ζ-potential. The apparatus was calibrated by Zeta Potential Transfer Standard (Malvern Instruments Ltd., Worcestershire, UK) with a known ζ-potential.

2.3.2. Platelet activation assay of Angiplex and pHPMA-MPPM polyplex in whole blood and in isolated platelets

In this assay, PE-anti-P-selectin and FITC-anti-fibrinogen were added to Angiplex, pHPMA-MPPM polyplex, bare lipoplex, and jetPEI polyplex with fluorescent marker conjugate to particle solution ratio of 1:25 and 1:100 (v/v), respectively. The buffer used was HBS buffer containing 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$ and 5 mM KCl at pH 7.4 and siRNA concentration was 1.1 μM. Platelets were isolated according to the method introduced by Korporaal *et al* [12]. In brief, blood was first centrifuged at 160 g in citrate anticoagulation tubes for 15 minutes at room temperature to obtain platelet-rich plasma. The number of platelets and the mean platelet volume (MPV) of the supernatant were determined by the Abbott Cell Dyne 1800 (Abbott diagnostic division, USA) and then the supernatant was treated with acid citrate dextrose solution (2.5% tri-sodium citrate, 1.5% citric acid, and 2% D-glucose) at a ratio of 1:10 (v/v). After another centrifugation step at 340 g for 15 minutes at room temperature, the pellet was resuspended in HEPES-Tyrode (HT) buffer (1.45 M NaCl, 50 mM KCl, 5 mM Na_2PO_4, 10 mM $MgSO_4$, and 100 mM HEPES) at pH 6.5. Prostacyclin was added to this solution at a ratio of 1:1000 (v/v). Then the solution was centrifuged at 340 g for 15 minutes at room temperature and the pellet was resuspended in 1 ml HT buffer at pH 7.2. The number of platelets and the MPV were measured to ensure that the blood platelets were not excessively activated and that the concentration was approximately the same as in the body. The MPV may only differ 1.5 fL and the blood platelet count was adjusted to 200,000 platelets per μl by adding more HT buffer at pH 7.2. Isolated platelets were left to rest for another 30 minutes before further use. Whole blood or isolated platelets were gently mixed with the particles at a ratio of 1:10 (v/v) and were incubated for 1, 10 or 20 minutes at room temperature. TRAP-6 which is a strong platelet activator was used as positive control of the assay. Thereafter, a

fixative solution (154 mM NaCl and 0.2% formaldehyde) was added to each sample with particle sample to fixative solution ratio of 1:20 (v/v). Fluorescence intensity of P-selectin (PE) and fibrinogen (FITC) was measured by flow cytometry with a FACS Canto II apparatus (BD Biosciences, San Jose, CA, USA) and analysed with FACSDiva™ software (BD Biosciences, San Jose, CA, USA). 10,000 events were recorded per sample.

2.3.3. Light transmission aggregometry in isolated platelets

Isolated platelets were prepared as described in section 2.3.2. 2 μl fibrinogen (25 mg/ml) was added to 500 μl isolated platelets and then different formulations (pHPMA-MPPM polyplex, jetPEI polyplex, Angiplex) were added to the platelet-fibrinogen mixture at the same volume. The final siVEGFR-2 concentration was 1.1 μM which was selected according to the highest clinical dose. JetPEI was used as a positive control particle and the complexes were prepared according to the manufacturer's protocol. Resting isolated platelets and HT buffer at pH 7.4 were used as negative controls. Then light transmission was measured by Model 700 Whole Blood/Optical Lumi-Aggregometer (Chronolog, UK).

2.3.4. Evaluation of contact system activation in the coagulation cascade

Particle solutions (Angiplex, pHPMA-MPPM polyplex, bare lipoplex and jetPEI polyplex), pooled plasma and L-2120 were pipetted in 96-well plates with volume ratios of 4:3:2, respectively, and optical density was measured in Spectramax 340 microplate reader (Molecular Devices, USA) at 405 nm. Kaolin (0.5 mg/ml) was used as control.

2.3.5. TLR activation assay

25,000 HEK-Blue™-hTLR2 reporter cells, 25,000 HEK-Blue™-hTLR3 cells, 12,500 HEK-Blue™-hTLR4 cells or 40,000 HEK-Blue™-hTLR9 cells were stimulated with 10% H_2O or Angiplex (at siRNA concentration of 0.1, 1, 10, and 100 pmol/ml) per well for 20 h at 37°C in a humidified CO_2-incubator. As positive controls, the following agonists were used: PAM3SCK (100 ng/ml) for TLR2, LPS-EK (10 ng/ml) for TLR4, ODN2006 (10 μg/ml) for TLR9 and Poly(I:C) (500 ng/ml) for TLR3 (all from Invivogen, Toulouse, France). To detect SEAP (reporter protein), 20 μl supernatant was added to 180 μl of QUANTI-Blue™ substrate (Invivogen, Toulouse, France) and incubated for 1 h at 37°C in a humidified CO_2-incubator. Levels of SEAP were determined with a Biorad microplate reader at 650 nm. Relative alkaline phosphatase levels were defined as sample level divided by water control level.

2.3.6. DC maturation assay

Bone marrow was flushed from femurs and tibia of female CB6F1/CrL mice (6-8 weeks). Femurs and tibia of adult mice were flushed with culture medium and cells were seeded at 3,000,000 cells per plate. Cells were expanded with 20 ng/ml murine rGM-CSF (Cytogen, the Netherlands) in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (Lonza, Verviers, Belgium), 50 µM 2-Mercaptoethanol (Sigma-Aldrich, St. Louise, USA), Penicillin and Streptomycin [13]. On days 2 and 5, additional 20 ng/mL rGM-CSF was added. On day 7, BMDCs were seeded in a 12-well plate at 2,000,000 cells/ml. After 6 h of resting, they were stimulated with PBS (1:100), LPS (10 ng/ml) or Angiplex (at siRNA concentration of 0.001, 0.01, 0.1, and 1 pmol/ml) for 16 h at 37°C in a humidified CO_2-incubator. Staining of surface markers with the indicated antibodies was performed in the presence of Fc block (2.4G2) for 30 minutes on ice. Anti-CD11c (N418) was purchased from eBioscience (San Diego, CA, USA) and anti-CD40 (3/23), -CD86 (GL1), and I-Ad/I-Ed (M5/114) were obtained from BD Biosciences (Breda, the Netherlands). Samples were measured on a FACSCantoII (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (Tree Star, OR, USA).

2.3.7. Complement activation assay

Whole blood samples of 6 healthy volunteers were collected in BD Vacutainer® silicon coated glass serum tubes with no additives (BD Biosciences, NJ, USA). Blood samples were allowed to clot at room temperature and centrifuged at 3,000 rpm for 5 minutes. Serum was collected and stored at -80°C which for usage was rapidly thawed at 37°C and placed on ice during the experiments. Complement activation was measured by Microvue SC5b-9 Plus ELISA kits. Sera were incubated with different lipoplex and polyplex formulations (4:1 v/v) for 30 minutes at 37°C in a shaking water bath. All tested formulations contained 1 µM siRNA. Zymosan (5 mg/ml) was used as positive control. After incubation, ELISA assays were performed according to the manufacturer's protocol. Absorbance was measured using BMG Labtech SpectroStarNano (BMG Labtech GmbH, Germany) at 450 nm. SC5b-9 concentrations were calculated using a linear curve fit. The percentage of increase in SC5b9 complex formation compared to the buffer indicated activation of the C system. Experiments were repeated twice. Statistical analysis was performed with GraphPad Prism 4 and two-tailed Student's t-test was used to calculate statistical significance.

3. Results and discussion

3.1. Preparation and characterisation of Angiplex and pHPMA-MPPM polyplex

Angiplex is a PEGylated lipoplex which has been used for targeted delivery of siRNA to tumor endothelial cells in our group (manuscript submitted for publication). The formulation consists of siRNA-protamine core encapsulated within a liposome composed of DOPE, CHEMS, DSPE-PEG2000-Mal, DSPE-PEG2000 at molar ratios of 6:4:0.3:0.3, respectively. Anginex peptide is coupled to the surface of the liposome to target galectin-1 receptors on endothelial cells of tumor vasculature. Angiplex has shown promising results in delivering siVEGFR-2 and has led to significant gene knock-down *in vitro* (manuscript submitted for publication). Another nanoparticle investigated in the current study was based on pHPMA-MPPM polymer which is a cationic and water-soluble polymer with a biodegradable linker that is stable in the endosome at pH 5 but is degraded at pH 7 in the cytosol [10]. pHPMA-MPPM siRNA polyplex has been reported to successfully silence firefly luciferase gene in H1299 human lung cancer cells expressing firefly luciferase *in vitro* [1]. Luten *et al.* administered complexes of pHPMA-MPPM with DNA i.p. in a Neuro2A tumor model in A/J mice. They observed poor gene expression in the tumor while the particles had mainly accumulated in the spleen [3]. These results suggest high clearance of these particles by the mononuclear phagocyte system which could also occur upon i.v. administration. Therefore, it seems crucial to evaluate nanoparticles in terms of potential interactions with blood components before continuing further with *in vivo* studies. In the current study, the carriers were loaded with siRNA as therapeutic payload.

To evaluate the effect of anginex peptide on the toxicity of lipoplexes, a bare lipoplex was prepared as control with the same composition as Angiplex excluding the peptide. As demonstrated in Table 1, pHPMA-MPPM polyplex had a size of around

Table 1 Mean size and mean zeta potential of pHPMA-MPPM polyplex, Angiplex and bare lipoplex (n=3)

Sample Name	Size (nm) ± SD	PDI	Zeta Potential (mV) ± SD
siVEGFR-2 pHPMA-MPPM polyplex	198 ± 12	0.3	25 ± 4
siVEGFR-2 protamine lipoplex (Bare lipoplex)	121 ± 5	0.2	-5 ± 1
siVEGFR-2 protamine anginex lipoplex (Angiplex)	150 ± 2	0.2	-5 ± 1

200 nm with a net positive charge. Angiplex and bare lipoplex were approximately 150 nm and 120 nm, respectively, with a net negative charge.

3.2. Angiplex activates platelets less than pHPMA-MPPM polyplex both in isolated platelets and in whole blood

Activation of platelets induced by Angiplex and pHPMA-MPPM polyplex was investigated by flow cytometry with PE-conjugated anti P-selectin monoclonal antibody and FITC-conjugated anti-fibrinogen polyclonal antibody. As a consequence of vascular injury, adhesive membrane proteins such as P-selectin (CD62p) relocate to the cell surface to further aid accumulation of platelets and aggregation [14]. Moreover, Von Willebrand Factor (vWF) and fibrinogen are released from secretion granules of platelets to form the crosslink between platelets in the aggregation phase [15]. Therefore, the level of these proteins on the surface of platelets can be used as indicators of platelet activation.

Assessing platelet behaviour towards nanoparticles in isolated platelets provides information about the direct effect of contact with these cells. Nevertheless, it could be more clinically relevant to evaluate interaction of nanoparticles with platelets in whole blood which is more similar to physiological conditions. It has been reported in the literature that blood components, red blood cells and white blood cells can have an impact on platelet activity [16]. Therefore, we investigated platelet activation for isolated platelets and in whole blood.

It has already been established that particles with a size between 100 and 300 nm can be recovered from the open canalicular system of platelets [17]. Since both particles investigated in this paper have a mean size around or less than 200 nm, their interaction with platelets is expected to be through this channel system. These interactions could induce signaling from the open canalicular system to the platelet membrane which could result in platelet activation.

Neither Angiplex nor bare lipoplexes induced significant expression of both types of activation markers be it in isolated platelets (Figure 1) or in whole blood (Figure 2). Levels of both fibrinogen (Figure 1A/2A) or P-selectin (Figure 1B/2B) remained low on the platelet surface over the 20 min incubation time. Bare lipoplex induced similar platelet activation as that of Angiplex which could indicate that the presence of anginex peptide did not have a significant impact on platelet activation. Accepted platelet activation threshold for a test sample is 20% of the positive control as stated by the Nanotechnology Characterization Laboratory (NCL) of the National Cancer Institute (http://ncl.cancer.gov/NCL_Method_ITA-2.pdf). Therefore, a general low platelet activation was concluded for Angiplex which can be attributed to the PEG molecules present in this formulation. PEG creates a hydrophilic shield around the particles. It has already been reported that more hydrophobic particles create higher platelet activation and aggregation [18].

In contrast, activation by pHPMA-MPPM polyplexes was prominent even compared to the positive control activator TRAP-6. Both for fibrinogen and P-selectin, surface expression was between one and over two orders of magnitude higher both in isolated platelets and whole blood. It is known that the effect of particles on the hemostatic system is mainly dependent on charge interactions. Since the surface of platelets is negatively charged by virtue of their sialic acid groups, positively charged particles such as pHPMA-MPPM polyplex are expected to interact with them via charge interactions. The mechanism behind this excessive activation could be through neutralizing the surface charge of platelets and consequently providing bridges between different platelets [19]. Therefore, we compared the activation pattern of pHPMA-MPPM to another cationic polymer, jet-PEI. The high levels of platelet activation, with a similar profile as for pHPMA-MPPM that were observed for jetPEI indicates that the positive charge could be responsible for the activation. There was a marginal effect of particle concentration on platelet activation in the siRNA concentration range from 0.5 to 10 µg/ml at different incubation times in both isolated platelets and whole blood (see Supplementary Figure 1, 2).

Of note is the observation that platelet activation was higher in general when the experiments were performed in isolated platelets than in whole blood. This could indicate that the particles interact directly with platelets rather than being dependent on opsonization by blood components. Another hypothesis, particularly in case of positive particles (such as pHPMA-MPPM polyplex), would be that interactions and surface adsorption of nanoparticles with those blood proteins which have a high negative charge density on their surface (such as albumin) have led to reduced platelet interaction and subsequent activation. Jones *et al.* have observed that due to such mechanism positively charged dendrimers, which activate isolated platelets *in vitro,* do not elicit platelet activation *in vivo* [20]. Another possible reason for the observed effect could be that the steps taken for isolating platelets have already primed these cells for activation [21]. Taken together, these results suggest that Angiplex is likely to evoke less platelet activation than pHPMA-MPPM polyplex *in vivo*.

3.3. Platelet aggregation is less affected by Angiplex than pHPMA-MPPM polyplex

In order to assess the effect of Angiplex and pHPMA-MPPM polyplex on platelet aggregation, an aggregometry assay was performed. Lower turbidity indicates more aggregation, since aggregation results in sedimentation of platelets and thus clearing up the sample within the light pathway. In line, with the FACS results on platelet activation, platelet aggregation was higher for pHPMA-MPPM polyplex (60%) than for Angiplex (30%) (Figure 3). Anginex peptide on the surface of Angiplex could be the reason for their higher aggregation compared to blank. JetPEI, which had shown a similar effect on platelet activation in FACS showed an intermediate aggregation

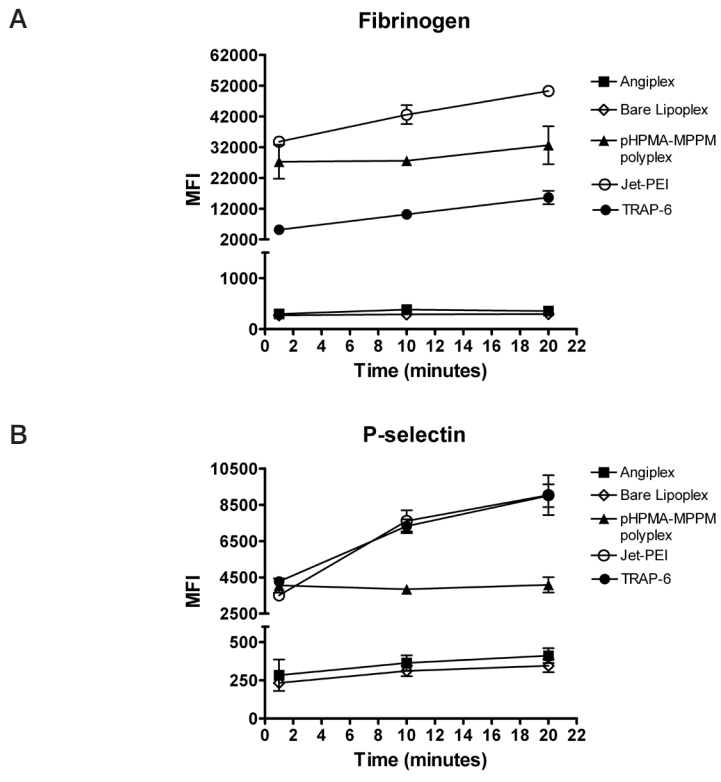


Figure 1 Platelet activation assay of Angiplex, bare lipoplex, pHPMA-MPPM polyplex, jetPEI polyplex, and TRAP-6 after incubation for 1, 10, and 20 minutes in isolated platelets represented by mean fluorescence intensity (MFI) of **(A)** FITC-conjugated anti-fibrinogen and **(B)** PE-conjugated anti P-selectin (n=3). All values are subtracted from blank.

(45%). The net positive charge of pHPMA-MPPM polyplex could have resulted in more interactions with platelets and therefore a rise in aggregation as it has already been reported in the literature that positively charged particles interact more strongly with platelets and thereby induce aggregation [22], [23].

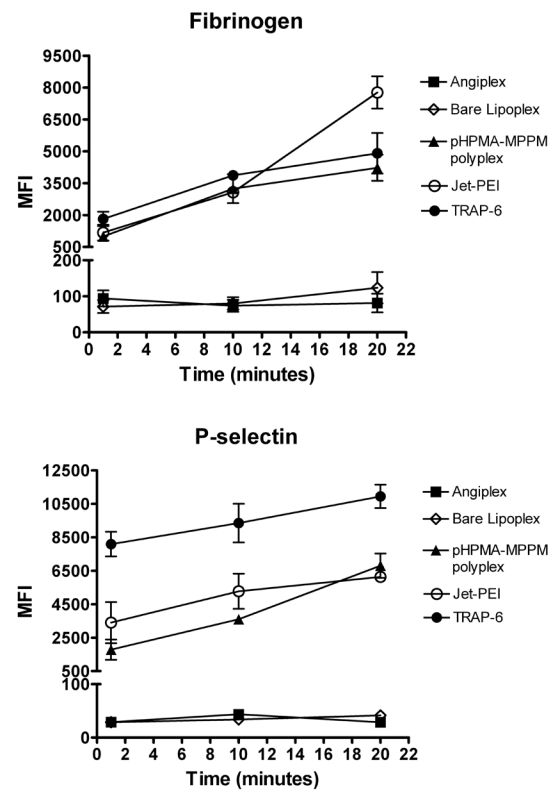


Figure 2 Platelet activation assay of Angiplex, pHPMA-MPPM polyplex, jetPEI polyplex, and TRAP-6 after incubation for 1, 10, and 20 minutes in whole blood represented by mean fluorescence intensity (MFI) of **(A)** FITC-conjugated anti-fibrinogen and **(B)** PE-conjugated anti-P-selectin (n=3). All values are subtracted from blank.

3.4. Contact system activation by Angiplex and pHPMA-MPPM polyplex is low

Impact of Angiplex and pHPMA-MPPM polyplex on initiating the intrinsic pathway of coagulation compared to a negatively charged particle, kaolin, which is employed as positive control was tested by contact system activation assay. Upon activation of the intrinsic pathway by nanoparticles, factor XII is autoactivated (denoted by XIIa). Subsequently, prekallikrein is activated which leads to the formation of kallikrein. In pooled plasma, the pathway stops at this stage because of the absence of calcium and phospholipids. In our assay, L-2120 was used as a substrate for kallikrein which

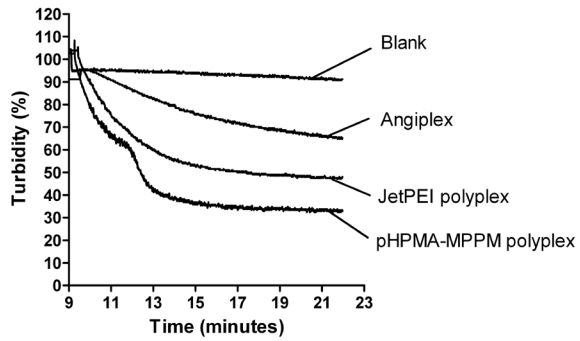


Figure 3 Platelet aggregometry assay of pHPMA-MPPM polyplex, jetPEI polyplex and Angiplex. % Turbidity is reversely correlated to the level of aggregation.

recognizes L-2120 sequence due to the analogy with its natural substrate. Thereafter, factor XIIa and kallikrein cleave the peptide between arg-pNa and pNa becomes a free molecule with a yellow colour. The colour is measured at 405 nm by a spectrophotometer.

Intrinsic pathway is activated by negatively charged surfaces such as kaolin [24]. As evidenced in Figure 4 (A, B), pHPMA-MPPM polyplex and jet-PEI had marginal effect on contact system activation compared to kaolin. This confirms previous findings that positively charged particles do not activate the contact system [16]. Miyamoto *et al.* have hypothesized that these particles are readily adsorbed to albumin (with a negatively charged surface). Another characteristic of particles contributing to coagulation is hydrophobicity [16]. This was in line with our observation that neither Angiplex nor bare lipoplex showed significant contact system activation (Figure 4 C, D). The absence of activation could be mediated by the PEG molecules on the surface of these particles which increase hydrophilicity. Moreover, although negatively charged liposomes are reported to trigger coagulation [25], PEG could have prevented this by shielding the negative charge.

3.5. Immunogenicity of Angiplex

Immunostimulation potential of Angiplex was determined by investigation of TLR2, 3, 4, and 9 activation on DCs and by evaluating expression of CD40, CD86 and MHC II in murine bone marrow-derived primary DCs. The immune system is tuned to react against invading pathogens. Foreign particles, often also therapeutic nanoparticles, are phagocytosed or endocytosed by antigen presenting cells (including DCs) which present them to T cells on their surface as a complex with MHCs. Various TLRs expressed in different cells recognize specific microorganism-associated molecular

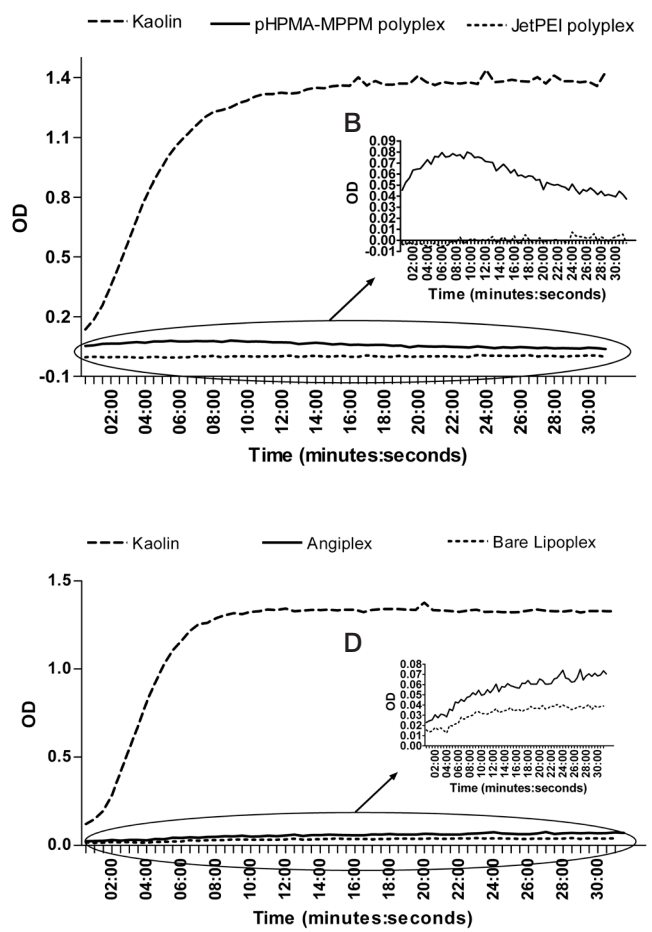


Figure 4 Contact system activation by **(A, B)** pHPMA-MPPM polyplex and jetPEI polyplex and **(C, D)** Angiplex and bare lipoplex expressed as the amount of L-2120 absorbance at 405 nm (OD: optical density). Graphs (B) and (D) are zoomed versions of graphs (A) and (C), respectively. In graphs (A) and (B), dash-dotted line represents jetPEI polyplex, solid line pHPMA-MPPM polyplex, and dashed line kaolin. In graphs (C) and (D), solid line represents Angiplex, dotted line bare lipoplex, and dashed line kaolin.

patterns (MAMPs). For instance, recognition sites for TLR2 are glycolipids, lipopolysaccharides (LPS) and lipopeptides on Gram-positive bacteria and yeast [26], bacterial LPS is recognized by TLR4 [27], [28], dsRNA by TLR3, and unmethylated CpG motifs by TLR9 [29], [30], [31]. Interaction of TLRs on DCs with MAMPs stimulates maturation of DCs which consequently activate T helper cells [32]. Moreover, expression of costimulatory molecules CD40 and CD86 in APCs is triggered by TLR signaling. Therefore, expression of CD86, CD40 and MHC II on these cells after encountering carrier systems was studied to investigate immunogenicity level of the nanoparticles.

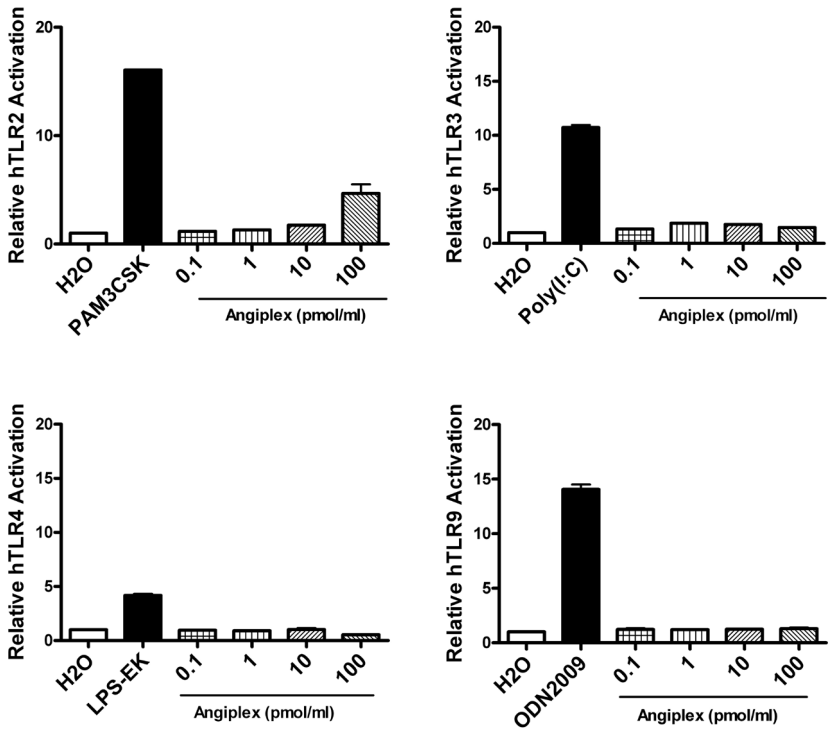


Figure 5 Effect of Angiplex on human TLR2, 3, 4, and 9 signaling pathways. Y-axes show relative TLR activation, i.e. ratio between SEAP secreted in test wells and that in H_2O control wells. Positive controls (PAM3CSK (100 ng/ml), Poly I:C (500 ng/ml), LPS-EK (10 ng/ml), ODN2009 (10 µg/ml)) are agonists of TLRs. TLR: Toll like receptor and SEAP: secreted embryonic alkaline phosphatase. Experiments were done in triplicates and the data are shown as mean ± SD.

As demonstrated in Figure 5, no significant activation of TLRs was observed after treatment with different concentrations of Angiplex. Furthermore, Angiplex did not stimulate DCs towards expression of CD40, CD86 and MHC II (Figure 6). This was in good agreement with results obtained by Vangasseri *et al.* who found that negatively charged lipids and fusogenic lipid DOPE have an insignificant stimulatory effect on expression of CD86 and CD80 by DC2.4 cells [33].

dsRNA is generally believed to trigger TLR3 which would result in the production of interferons, tumor necrosis factor alpha and interleukin-6 [34], [35]. However, our results showed only marginal effect of Angiplex on TLR3 activation which is most likely due to sequestering the siRNA within the formulation so that it is not accessible to these receptors. The implications from the above findings are that Angiplex does not have a major immunostimulatory capacity which would render it suitable for *in vivo* studies.

3.6. Complement activation assay

Activation of the C system was assessed by determining the amount of SC5b-9 complex formation in sera of 6 individuals. It has been well established that anionic liposomes can activate the C system via the classical pathway [36]. However, our results showed that Angiplex did not trigger complement activation to a significant extent (Figure 7) which could be due to the very low overall negative charge of these particles, PEG shielding effect, and the presence of the neutral lipid DOPE in the formulation which has been reported in other studies to reduce activation of the C system once included in DNA-lipid complexes [37]. Bare lipoplexes caused higher activation of the C system than Angiplex (Figure 7). As this effect was absent when bare lipoplexes were prepared with DSPE-PEG2000 instead of DSPE-PEG2000-Mal or when sera were incubated with Angiplex (which has little reactive maleimide groups left after the coupling), the result was concluded to be related to free maleimide groups. Maleimide groups are quite reactive and could have reacted with complement proteins. Low activation was observed with pHPMA-MPPM polyplex and jetPEI polyplex which was consistent with the study of Plank *et al.* who showed that although positively charged polymers have the potential to activate the C system, complexation with nucleic acids decreases activation because less positive charges would be accessible for interaction with the complement proteins [38]. Moreover, complement activation by polyplexes was lower than lipoplexes. This is plausibly a consequence of including DSPE-PEG2000 in the formulation of lipoplexes. DSPE-PEG2000 in the formulation of liposomes has a dual effect; on the one hand it shields the charge and on the other hand it has been shown in the literature to increase C activation by liposomes likely due to the negatively charged phosphate moieties [39].

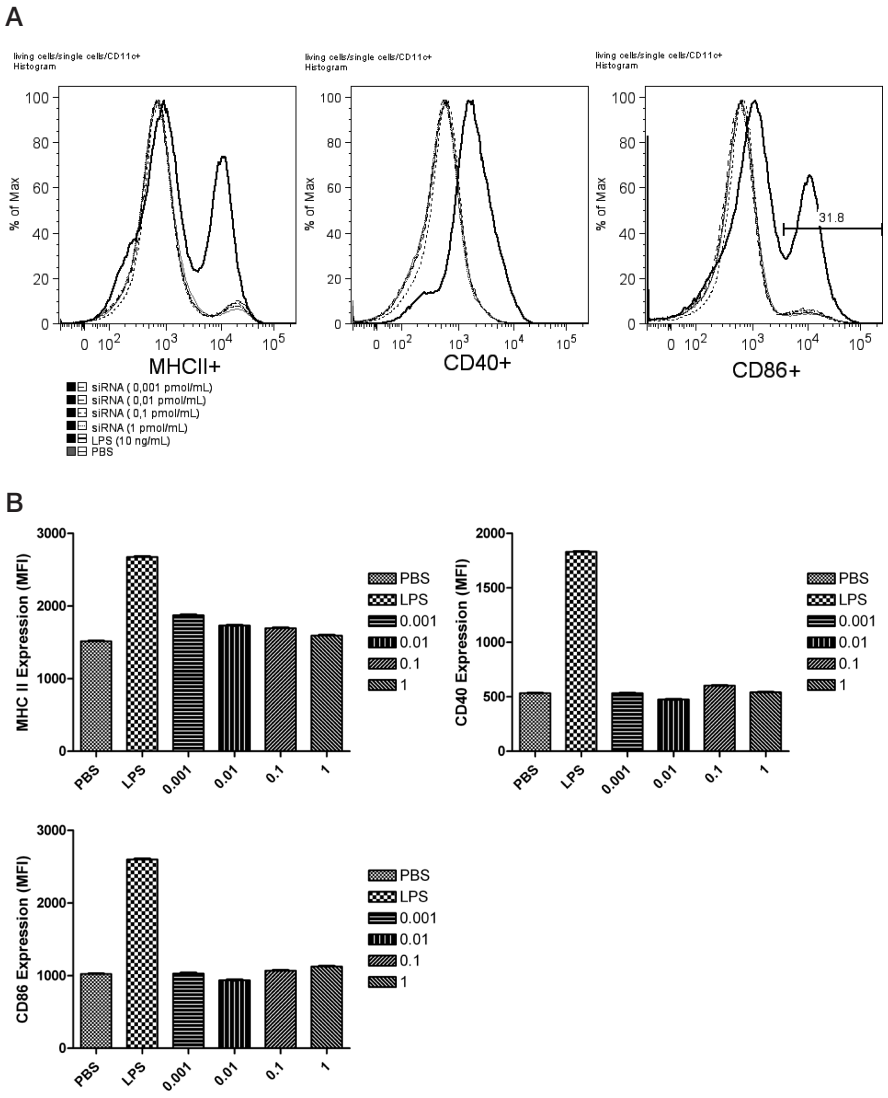


Figure 6 **(A)** Expression of surface markers of bone marrow derived DCs by flow cytometry analysis. DCs were stimulated 16 h with PBS, LPS (10 ng/mL) or Angiplex (0.001, 0.01, 0.1, or 1 pmol/mL). LPS: lipopolysaccharide. Maturation was measured by the surface markers MHC-II, CD40, and CD86. **(B)** Expression of surface markers of bone marrow derived DCs represented by mean fluorescence intensity (MFI). Data represent mean ± SEM.

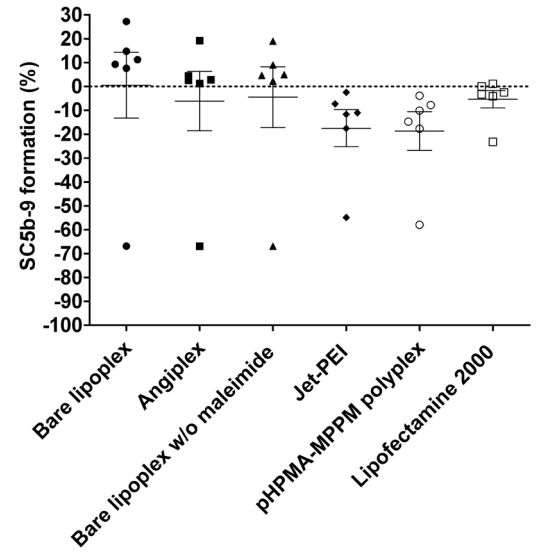


Figure 7 Formation of SC5b-9 after incubation of human serum with lipoplexes and polyplexes. Results are given as % of SC5b-9 formation normalized to 20 mM HEPES buffer (non-activator) and zymosan (100% level of activation). Black dots indicate the mean of two measurements in an individual serum and error bars represent SEM.

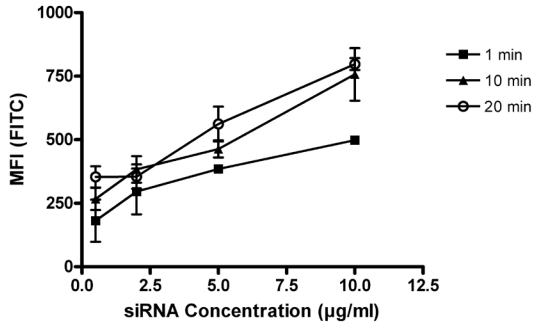
4. Conclusion

Thrombogenicity, plasma coagulation, and complement activation are among the most commonly used *in vitro* hemocompatibility tests for nanomedicine according to NCL (http://ncl.cancer.gov/). We investigated the behavior of a lipoplex (Angiplex) and a polyplex (prepared with pHPMA-MPPM) in the above tests as well as the immunogenicity of Angiplex. We found that interactions depend on physicochemical properties of the nanoparticle and that each nanoparticle is affected differently by various components and cells in the blood. Based on the NCI criteria, Angiplex did not significantly stimulate platelet activation and aggregation, while pHPMA-MPPM and jetPEI polyplex had a strong stimulatory effect on platelets likely due to their net positive surface charge. Both formulations showed minimal activation of the C system. Also both formulations did not activate the contact system of coagulation significantly and Angiplex had minimal impact on immune stimulation via the TLRs. Overall, these results suggest that Angiplex is expected to cause minimal adverse

effects in the blood stream, whereas pHPMA-MPPM is likely to lead to complications due to stimulating platelets. Taken together, the assays performed in this study allow us to obtain a comprehensive overview of nanoparticle interactions with the blood from different aspects. This can be an essential step preceding *in vivo* administration in order to decrease animal usage and suffering.

5. Supplementary Material

A



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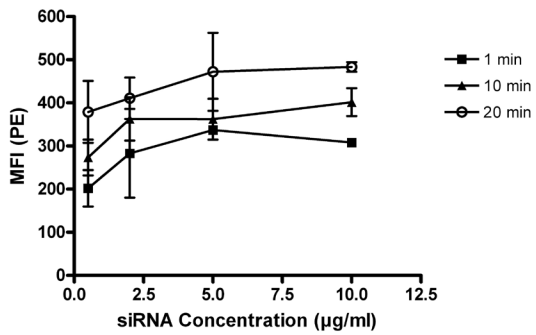
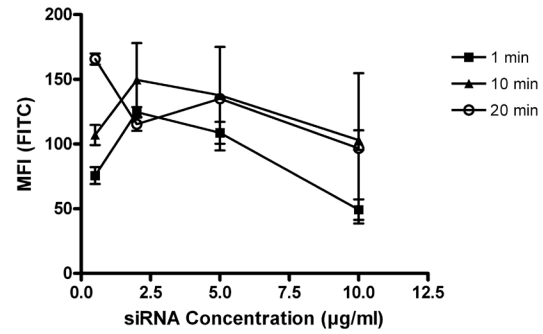


Figure 1 Effect of concentration of lipoplexes on platelet activation after 1, 10, and 20 minutes of incubation with isolated platelets represented by mean fluorescence intensity (MFI) of **(A)** FITC-conjugated anti-fibrinogen and **(B)** PE-conjugated anti P-selectin (n=3). siRNA concentrations were 0.5, 2, 5, 10 µg/ml. All values are subtracted from blank.

A



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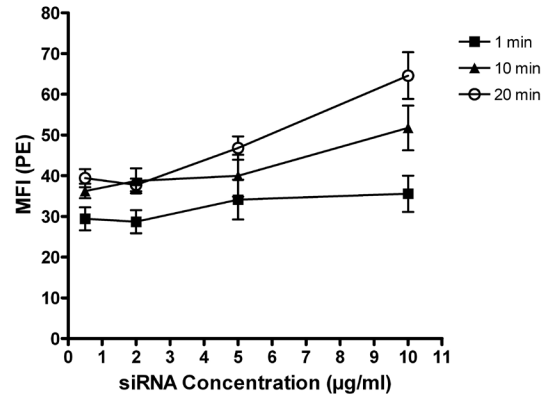

Figure 2 Effect of concentration of lipoplexes on platelet activation after 1, 10, and 20 minutes of incubation with whole blood represented by mean fluorescence intensity (MFI) of **(A)** FITC-conjugated anti-fibrinogen and **(B)** PE-conjugated anti P-selectin (n=3). siRNA concentrations were 0.5, 2, 5, 10 µg/ml. All values are subtracted from blank.

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6

Preliminary results on the biodistribution and anti-angiogenic efficacy of systemically administered anginex lipoplexes in a murine neuroblastoma tumor model

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Abstract

In the treatment of cancer, parallel to chemotherapeutics, great attention has been drawn to inhibiting the formation of new vasculature (angiogenesis) in tumors in order to limit their supply of oxygen and nutrients. Current anti-angiogenic therapeutics, however, are not satisfactory due to severe side-effects. Therefore, there is a need in the field for more effective alternatives. Silencing gene expression of proteins involved in angiogenesis by siRNA seems an attractive approach. One of the most important pro-angiogenic proteins is vascular endothelial growth factor (VEGF). Upon interaction with its main receptor (VEGFR2) on tumor endothelial cells, VEGF leads to a series of signaling cascades which result in angiogenic events such as endothelial cell migration and proliferation. Therefore, application of siRNA silencing the expression of VEGFR2 could be used for anti-angiogenic purposes. Previously, we have reported on successful knock down of VEGFR2 gene in human umbilical vein endothelial cells (HUVECs) *in vitro* after treatment with the targeted anginex-lipoplex delivering siVEGFR2 (Angiplex) (chapter 4). In the current study, we have investigated biodistribution of Angiplex containing siRNA against siVEGFR2 and evaluated its efficacy in tumor growth inhibition *in vivo*. Our preliminary results showed that Angiplex maintained the siRNA while circulating in the blood up to 48 h while somewhat higher siRNA was observed in the lungs and spleen than in the tumor or other organs. We did not observe significant tumor growth inhibition after *i.v.* administration of Angiplex in mice.

1. Introduction

One of the major challenges in medicine at the moment is controlling and treating cancer. Apart from conventional chemotherapeutics, drugs influencing the tumor vasculature have also drawn much attention especially in combination with chemotherapy [1], [2]. Rapid cell division in tumors makes it vital that, after the tumor size exceeds a critical size (~1-2 mm^3), new blood vessels are formed from pre-existing capillaries, a phenomenon known as angiogenesis [3]. In response to lack of oxygen and nutrients, angiogenic growth factors are secreted from tumor cells which interact with receptors on the endothelial cells (ECs) of pre-existing vasculature. Activation of these quiescent ECs leads to their migration, proliferation, and maturation to neo-vasculature [4]. Angiogenesis is controlled by the balance between pro- and anti-angiogenic factors which is shifted towards the pro-angiogenic elements in tumor growth [3]. Among the different angiogenesis regulators such as platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietins, VEGF is one of the most well-studied. It contributes to angiogenesis by being involved in endothelial cell proliferation, migration, and vascular permeability [5], [6]. This protein can bind to several receptors (VEGFR1, VEGFR2, and VEGFR3) among which VEGFR2 is highly expressed in vascular ECs and is implicated in angiogenesis [7]. Therefore, inhibiting the function or expression of this receptor can assist in the suppression of tumor growth. One strategy to reach this goal is by using siRNA against the gene coding for VEGFR2. siRNA is a small RNA composed of 21-23 nucleotides which is a key player in RNA interference (RNAi). However, siRNA need to be delivered with a carrier in order to be used as a systemic therapeutic [8]. This is due to susceptibility of small RNAs to degradation by nucleases in the blood and being negatively charged which makes cell uptake difficult. Although many viral and non-viral delivery systems for siRNA have been investigated so far, successful *in vivo* delivery still remains a challenge. Viral systems offer high efficiency but their application is limited as a result of their oncogenic and immunogenic side-effects. Non-viral vectors can be designed in a way to trigger minimum immune response but usually have low efficiency compared to viral carrier systems. In this study, we explored a non-viral delivery system to circumvent safety issues. Our delivery system, anginex-lipoplex (Angiplex) is a negatively charged PEGylated complex with low toxicity and significant gene silencing activity in HUVECs *in vitro* (chapter 4). It has also been shown to trigger low platelet activation, aggregation, coagulation, and complement activation in human serum (chapter 5). Moreover, immune response stimulation of this system has been reported to be minimal (chapter 5). Anginex, a 33-mer peptide, has been included in the formulation as a targeting ligand because of its high affinity for galectin-1 receptors which are present abundantly on tumor

endothelial cells [9], [10]. In the current study, we have investigated the biodistribution of Angiplex containing siVEGFR2 after i.v. injection in a neuroblastoma tumor model in mice as well as the efficacy of this system in preventing tumor growth *in vivo*.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)] with PEG Mw of 2000 g.mol-1 was obtained from Lipoid (Ludwigshafen, Germany). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-Mal) were purchased from Avanti® Polar Lipids Inc. (Alabaster, USA). Cholesteryl hemisuccinate (CHEMS), protamine sulphate salt, and hematoxylin were supplied by Sigma-Aldrich (St. Louise, USA). siRNA against human VEGFR2 (siVEGFR2) with the sequence: sense strand: 5´-CCG-GAA-AUC-UGG-AGA-AUC-ATT-3´, anti-sense strand: 5´-UGA-UUC-UCC-AGA-UUU-CCG-GTT-3´ and negative control scrambled siRNA (NC siRNA) with the sequence: sense strand: 5´-CAU-CGU-CGA-UCG-UAG-CGC-ATT-3´, anti-sense strand: 5´-UGC-GCU-ACG-AUC-GAC-GAU-GTT-3´ were obtained from Eurogentec Nederland B.V. (Maastricht, the Netherlands). Alexa Fluor 488 (AF488) labelling at the 3´ end of the sense strand of siVEGFR2 and NC siRNA was performed by Eurogentec Nederland B.V. (Maastricht, the Netherlands).

Protected N-terminal SATA-modified anginex peptide with the sequence: SA-TA-ANIKLSVQMKLFKRHLKWKIIVKLNDGRELSLD and bpeptide-28 (bpep-28) with the sequence: SATA-SIQDLNVSMKLFRKQAKWKVIVKLNDGRELSLD were provided by ChinaPeptides Co., Ltd. (Shanghai, China). Anti-CD31 primary antibody and Goat Anti-Rabbit IgG H&L (HRP) secondary antibody were purchased from Abcam (Cambridge, USA). Cyclic 5-mer RGD (c(RGDf(e-S-acetylthioacetyl)K) was synthesized by JPT Peptide Technologies (Springfield, USA). All the other reagents were of analytical grade.

2.2. Cell culture

Murine neuroblastoma cells (Neuro2A ; ATCC CCL-131) were cultured in RPMI 1640 medium which was supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. The cells were maintained in a humidified CO_2-incubator at 37°C.

2.3. Preparation and characterization of lipoplexes

Protamine-siVEGFR2-anginex-lipoplex (pt-siVEGFR2-Ax-lipoplex), protamine-NC siRNA-anginex-lipoplex (pt-NC siRNA-Ax-lipoplex), protamine-siVEGFR2-bpep-tide-28-lipoplex (pt-siVEGFR2-bpep-28-lipoplex), and protamine-siVEGFR2-RGD-lipoplex (pt-siVEGFR2-RGD-lipoplex) were prepared by the following method: DOPE, CHEMS, DSPE-PEG2000, and DSPE-PEG2000-Mal were mixed at molar ratios of 6:4:0.3:0.3, respectively, and dissolved in chloroform:ethanol (3:4 v/v) on a rotavapor (Buchi, Switzerland) at 40°C. A lipid film was then made by evaporation of the solvents under vacuum. siRNA was complexed with protamine (pt) at w/w ratio of 1:1.2 in 20 mM HEPES buffer containing 5% glucose at pH 7.4 (HBG). The mixture was vortexed and incubated at room temperature for 20 minutes. Thereafter, this mixture was added to the lipid film which was then hydrated on rotavapor at 40°C to form lipoplexes. Lipoplexes were then extruded repeatedly through polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) to a size of about 100 nm by a Lipex™ Extruder (Northern Lipids, Burnaby, BC, Canada). Total lipid concentration was 10 mM determined by method developed by Rouser *et al*. [11]. Final siRNA concentration was 15.2 nmol/ml and 51% encapsulation efficiency was determined by Ribogreen assay (Invitrogen, Breda, the Netherlands) according to the manufacturer's protocol. Deprotecting of peptides (anginex, bpep-28, or cRGD) were performed by mixing them with a deprotecting solution (0.05 M HEPES, 0.05 M hydroxylamine, and 0.03 mM EDTA at pH 7.0) at a peptide to deprotecting solution ratio of 1:10 (v/v). After incubation at room temperature for 45 minutes, the mixture was added to lipoplexes and incubated over night at 4°C. In order to remove the uncoupled peptides, ultra-centrifugation was performed at 200,000 g for 1 h at 4°C. Concentration of peptides was calculated as 10 µg anginex or bpep-28 per µmol phospholipid and 4 nmol cRGD per µmol phospholipid assuming 100% coupling.

Dynamic light scattering was used to measure the hydrodynamic diameter of lipoplexes. Measurements were performed at 25°C on a Malvern 4700 system using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern Instruments Ltd., Worcestershire, UK). Viscosity and refractive index of water at 25°C and a 90° angle were applied in all the measurements. The system was calibrated by standard polystyrene beads with a diameter of 200 nm (Thermoscientific, DE, USA). Size distribution was denoted by PDI (polydispersity index) with a range from 0 (monodisperse sample) to 1 (polydisperse sample). Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, UK) was used for determining ζ- potential at 25°C. Zeta Potential Transfer Standard (Malvern Instruments Ltd., Worcestershire, UK) with a known ζ- potential was used for calibrating the instrument.

2.4. Biodistribution study in a Neuro2A tumor model in A/J mice

Six to nine-week old male A/J mice (Harlan, the Netherlands) with weight of 20 - 25 g were inoculated subcutaneously in their right flank with 100 µl Neuro2A cells (1 x 10^6 cells/ml) in PBS. Tumor dimensions were measured by digital calipers and tumor size

was calculated according to the following formula: Size = length x width^2 x π/6. Two hundred micro liters of formulation (pt-siVEGFR2-Ax-lipoplex, pt-NC siRNA-Ax-lipoplex, pt-siVEGFR2-bpep-28-lipoplex) or naked siVEGFR2 was injected i.v. in the tail vein at tumor volume of approximately 200 mm^3. The control group did not receive any formulation. Concentration of siRNA per injection was 5 mg/kg. All groups consisted of 3 mice. Animals were sacrificed by CO_2/O_2 inhalation at 15 min, 60 min, and 48 h after injection. Blood was collected in Heparin/EDTA-tubes and plasma was separated by centrifugation at 2500 rpm for 5 minutes at 4°C. Organs (liver, spleen, lungs, kidneys) and tumors were snap frozen in liquid nitrogen. The Dutch national regulations, "Wet op de dierproeven" (art. 9) (1977), were followed in all the animal experiments and the protocols were approved by the local animal experiments ethical committee.

For histology and fluorescence microscopy imaging, 5 µm sections were prepared by a cryostat microtome (Leica, Wetzlar, Germany). Images were taken from the sections with a Nikon TE2000-U fluorescent microscope (Nikon Benelux, Brussels, Belgium) equipped with a GFP filter at X 100 magnification. For histology studies, after fixation of sections in acetone, they were incubated in anti-CD31 primary antibody (diluted 1:50) followed by Goat Anti-Rabbit IgG H&L (HRP) secondary antibody (diluted 1:1000). Consequently, 3,3'-diaminobenzidine (DAB) was used for staining and hematoxylin was used for counterstaining. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide. Images from slides were taken with a Nikon TE2000-U microscope (Nikon Benelux, Brussels, Belgium) at X 100 magnification.

2.5. siRNA quantification by Stem-Loop RT PCR

Total RNA was isolated from plasma, organs (liver, spleen, lungs, kidneys), and tumors by TRIzol® (Life Technologies, NY, USA) according to the manufacturer's protocol and RNA concentration was determined by Nanodrop (Thermoscientific, DE, USA). Stem-Loop RT PCR (SL-RT PCR) was performed on RNA isolated from all plasma samples as well as that of tumors and organs (liver, spleen, lungs, kidneys) of 15 min and 48 h samples for detecting siVEGFR2 according to method developed by Chen *et al* [12]. SL-RT PCR experiments were performed by Applied Biosystems Veriti 96-w Thermal Cycler and BioRAD CFX Real-Time PCR Detection System. All experiments were normalized for U6 transcript expression. Sequences of the primers were as follows:

siVEGFR2 anti-sense strand:
Stem-loop primer:
5´-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCGG-3´
Specific forward primer: 5´-GCCCGCTGATTCTCCAGATTTCC-3´
Universal reverse primer: 5´-GTGCAGGGTCCGAGGT-3´

U6:
Stem-loop primer: 5´-GTCATCCTTGCGCAGG-3´
Specific forward primer: 5´-CGCTTCGGCAGCACATATAC-3´
Specific reverse primer: 5´-AGGGGCCATGCTAATCTTCT-3´
Statistical analysis was performed by GraphPad Prism 4 software using a one-way ANOVA with a Bonferroni post-test to calculate statistical significance.

2.6. Efficacy study in a Neuro2A tumor model in A/J mice

Six to nine-week old male A/J mice weighing 20 - 25 g were provided by Harlan (the Netherlands). Animals were divided in groups of 3 mice and 100 µl Neuro2A cells (1 x 10^6 cells/ml) in PBS were inoculated subcutaneously in their right flank. Tumor dimensions were measured every second day by digital calipers and tumor size was calculated as described in section 2.4. Two hundred micro liters treatment (PBS, pt-siVEGFR2-Ax-lipoplex, pt-NC siRNA-Ax-lipoplex, pt-siVEGFR2-RGD-lipoplex) was injected i.v. in the tail vein at tumor volume of approximately 200 mm^3. Concentration of siRNA per injection was 5 mg/kg. Injections were performed on days 0, 2, 4, 6, and 8. Mice were sacrificed by CO_2/O_2 inhalation when the tumor size reached the human end point (1500 mm^3). The Dutch national regulations, "Wet op de dierproeven" (art. 9) (1977), were followed in all the animal experiments and the protocols were approved by the local animal experiments ethical committee.

3. Results and discussion

3.1. Preparation and characterization of lipoplexes

Lipoplexes were composed of a protamine-siRNA core which was complexed within a liposomal structure. Protamine is commonly used to increase encapsulation efficiency of siRNA in neutral or negatively charged carriers [13], [14], [15]. In order to provide the particles with endosomal escape properties, DOPE was included in the composition of the lipid bilayer. DOPE is a fusogenic lipid which creates negative curvature at acidic pH leading to the transition of the phospholipid bilayer from lamellar (L_α) to hexagonal phase (H_{II}) and fusion with the endosome membrane [16], [17]. Since these particles were aimed to be administered *i.v.*, DSPE-PEG2000 was used to generate a hydrophilic shield on the surface of the particles protecting them from being readily recognized by macrophages and delaying their clearance [18]. It is noteworthy that PEG has the potential to decrease the fusogenic properties of DOPE [19], [20] but since it has an intrinsic fusogenic property itself, the net effect would be favorable [21]. Characterization of lipoplexes showed that they were monodisperse and possessed an approximate size of 128 nm (Table 1). Their net negative charge of around -9 mV (Table 1) makes them favorable over cationic

carriers for *in vivo* applications because of causing less interactions with plasma proteins [22], [23], [24]. However, negative surface charge of nanoparticles can hinder their uptake by target cells. Therefore, a targeting ligand (anginex) was coupled to the distal end of PEG chains in DSPE-PEG2000-Mal in order to facilitate uptake by ECs of tumor vasculature. Anginex has high affinity for galectin-1 receptors which are expressed on ECs of tumor vasculature [9], [10] and has already been shown to result in specific internalization of Angiplex in HUVECs *in vitro* (chapter 4).

Table 1 Mean size and mean zeta potential of lipoplexes (n=3)

Sample Name	Average Size (nm) ± SD	PDI	Average Zeta Potential (mV) ± SD
siVEGFR2 protamine Ax lipoplex (Angiplex)	123 ± 3	0.1	-8.3 ± 0.9
siVEGFR2 protamine bpep-28 lipoplex (bpep-28-L)	135 ± 2	0.1	-8.5 ± 0.4
NC siRNA protamine Ax lipoplex	128 ± 2	0.1	-9.19 ± 0.5
siVEGFR2 protamine RGD lipoplex	125 ± 5	0.1	-10 ± 0.8

3.2. Angiplex maintains the siRNA in the circulation

To investigate biodistribution of Angiplex, tumor bearing mice were treated with Angiplex, siVEGFR2 protamine bpep-28 lipoplex, NC siRNA protamine Ax lipoplex, and naked siVEGFR2 intravenously. Plasma samples were taken at 15 min, 60 min, and 48 h after injection and were analyzed by SL-RT PCR to detect the presence of siVEGFR2. SL-RT PCR was selected as a detection method instead of more common methods such as tracking radio- or near infrared-labeled siRNA because it would give us direct evidence that the siRNA has been intact. As depicted in Figure 1, considerable amounts of siVEGFR2 was detected in plasma through time for Angiplex and siVEGFR2 protamine bpep-28 lipoplex compared to non-treated mice. Moreover, naked siVEGFR2 appeared to have degraded or been cleared rapidly already at 15 min. These observations indicated that the formulation was stable up to 48 h after injection which would provide sufficient time for it to encounter target cells increasing the chance of ligand-receptor binding and uptake. In addition, it maintained the siRNA intact which is necessary for its functionality.

3.3. Biodistribution of Angiplex

In order to investigate distribution of particles in different organs and the tumor, AF488-labeled siVEGFR2 was used in the biodistribution study and fluorescence of

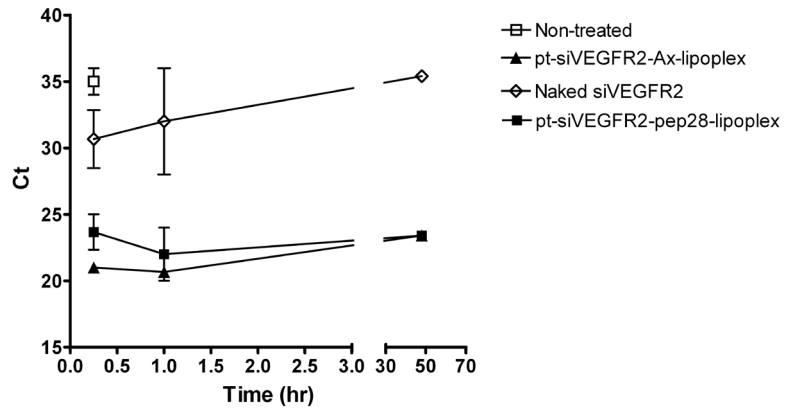

Figure 1 SL-RT PCR of plasma samples showed presence of pt-siVEGFR2-Ax-lipoplex (Angiplex) in circulation. X-axes presents Ct values and Y-axes presents time of blood collection after injection (n=3).

tissue sections was evaluated by fluorescent microscopy. A strong autofluorescence was observed in all the organs and tumors including the non-treated mice which prevented any quantification (data not shown). Therefore, we used an alternative method, SL-RT PCR, to assess the presence of siVEGFR2 in the tumor, lungs, kidneys, liver, and spleen of mice which were sacrificed at 15 min and 48 h after injection. All experiments were normalized for U6 transcript expression. As demonstrated in Figures 2A and 2B, siVEGFR2 levels were higher in the lungs and spleen at the 15 min time point in tissue samples of mice treated with Angiplex compared to bpep-28-targeted formulations or naked siVEGFR2. This observation could be because of the high vascularization of the lung tissue which would result in presence of relatively large amounts of blood. Since our detection method could not discriminate between siRNA present in the blood and siRNA in the tissue, it could be plausible that more siRNA is detected in a better-vascularized organ.

As depicted in Figures 2A and 2B, less siRNA was detected in the lungs of mice treated with siVEGFR2 protamine bpep-28 lipoplex compared to those treated with Angiplex which could be due to interaction with galectin-1 receptors. Although galectin-1 over expression is mainly observed in tumors, the vast endothelium of the lungs might have led to the presence of more galectin-1 in the lung tissue than other organs. However, it is expected that the same effect should be observed in the tumors which over express galectin-1 but according to our results, this did not occur. Since the specificity of Angiplex had already been confirmed *in vitro* (chapter 4), we hypothesized that anginex has lost its biological activity *in vivo* likely due to a change

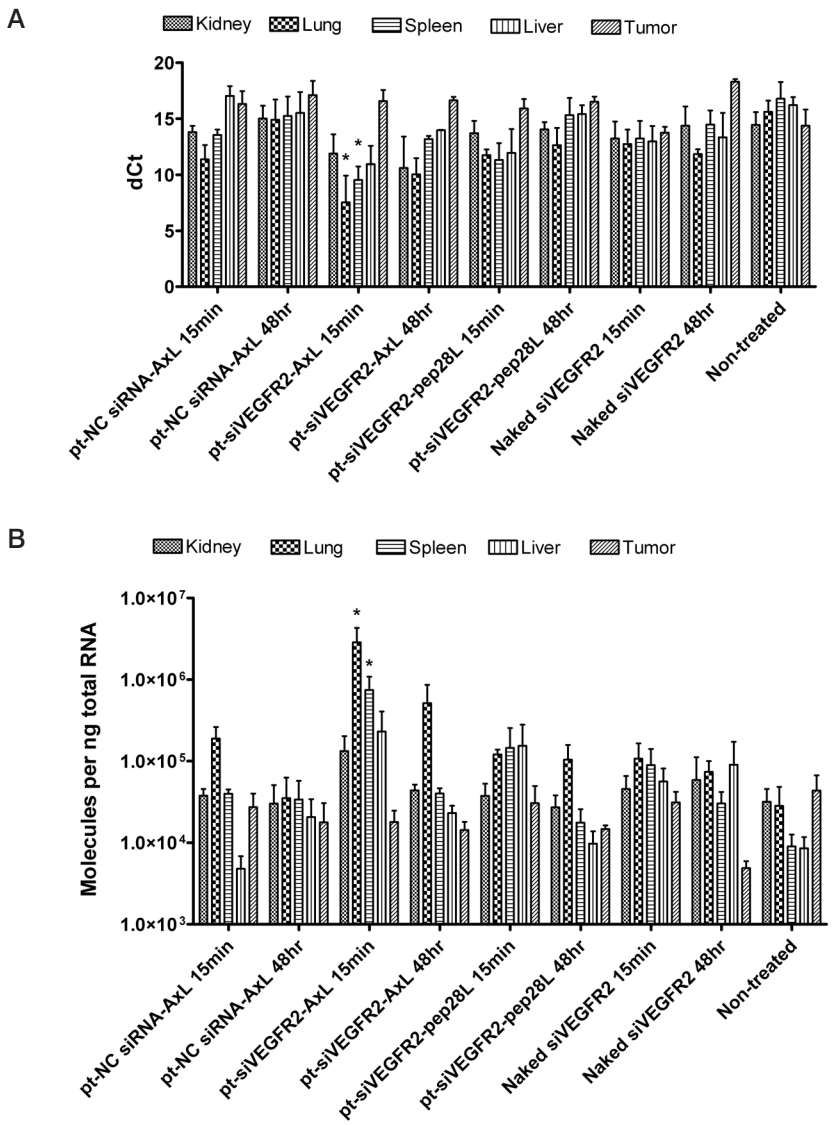

Figure 2 Presence of lipoplexes in tumor, kidney, liver, spleen, and lung of 15 min and 48 h samples was determined by SL-RT PCR for siVEGFR2. Treatments consisted of Angiplex (pt-siVEGFR2-AxL), siVEGFR2 protamine bpep-28 lipoplex (pt-siVEGFR2-pep28L), NC siRNA protamine Ax lipoplex (pt-NC siRNA-AxL), and naked siVEGFR2. Results are presented as mean dCt ± SD **(A)** and mean number of siRNA molecules per ng total RNA ± SD **(B)**. $p<0.05$ is denoted by * (n=3). All data are normalized for U6 expression.

in conformation. Another possible reason could be that Angiplex has interacted with galetin-1 present on activated tissue macrophages [25] which has led to sequestration of anginex from endothelial cells of tumor vasculature.

Presence of siVEGFR2 at 15 min after injection of Angiplex in the spleen indicates that the particles have been phagocytosed which could be related to the surface charge of particles. It has been reported in the literature that anionic liposomes are taken up by spleen more than neutral liposomes [26]. This could be overcome in future experiments by increasing the PEG density to shield the charge of particles more since not only the presence of PEG but more importantly the PEG density determines pharmacokinetics of nanocarriers [27].

Absence of siRNA in mice treated with naked siVEGFR2 at both time points is likely due to degradation or rapid elimination which is in line with the data from blood circulation (Figure 1). Slight amount of siVEGFR2 detected in mice treated with NC siRNA protamine Ax lipoplex (15 min time point) could be a result of similarities between the two sequences.

Although by using SL-RT PCR, we acquired information on the presence of siVEGFR2, methods such as radio-labelling the particles need to be performed in complementary experiments in order to draw strong conclusions about accumulation in different organs and the tumor.

3.4. No macroscopic anti-angiogenic effect is observed with Angiplex

Since microvessel density is a measure of tumor angiogenesis [28], immunohistochemical CD31 staining of microvessels in tumors from the 48 h time point was performed (Figure 3). CD31, or platelet endothelial cell adhesion molecule-1, is abundantly found on the surface of ECs and is involved in the adhesion of adjacent ECs during angiogenesis [29]. Therefore, it is widely used for quantification of microvessel density in angiogenic tumors [30], [31]. Three to five areas were selected at X 100 magnification per tumor and the number of vessels was quantified (Figure 4). As indicated in Figures 3 and 4, no significant difference in microvessel density was observed between the treatment groups. This observation was not unexpected because only a single injection was performed in this study since the main goal was to track distribution of particles and not their efficacy. Generally, in order to see biological outcome of silencing (i.e. efficacy studies) multiple dosing of siRNA is necessary because of its transient and short-time effects.

3.5. Anti-tumor efficacy of Angiplex

In order to test the anti-angiogenic effect of Angiplex *in vivo*, A/J mice bearing Neuro2A tumors were injected intravenously with Angiplex, siVEGFR2 protamine RGD lipoplex, NC siRNA protamine Ax lipoplex, or PBS. Each injection contained 5 mg/kg siVEGFR2 or NC siRNA and a total number of 5 injections were performed

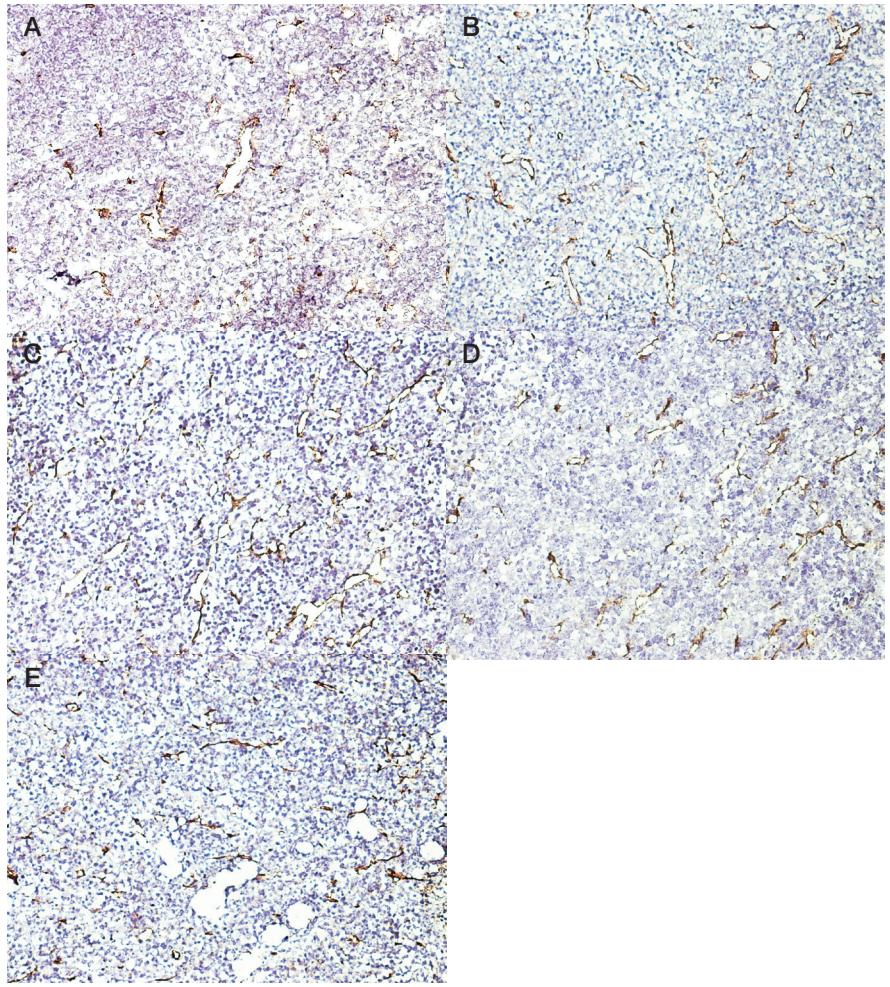

Figure 3 CD31 staining of tumor sections of mice which were sacrificed 48 h after injection; **(A)** Non-treated, **(B)** Angiplex, **(C)** Pt-siVEGFR2 pep28-lipoplex, **(D)** Naked siVEGFR2, **(E)** Pt-NC siRNA Ax-lipoplex.

every second day. Monitoring the tumor size showed no significant tumor growth inhibition after treatment with Angiplex compared to control formulations (Figure 5). This could be related to low presence in the tumor as stated in the biodistribution study (section 3.3). Also, since no effect was observed for positive control formulation (pt-siVEGFR2-RGD-lipoplex) either, it could indicate that the set-up of the experiment

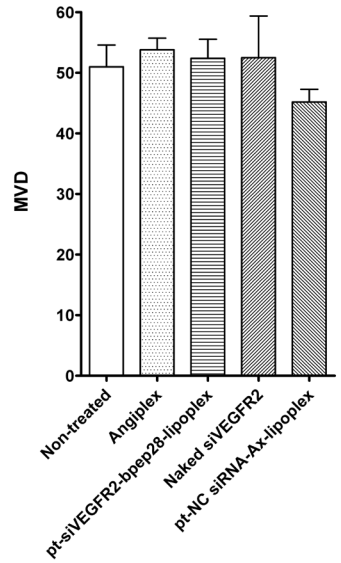

Figure 4 Microvessel density (MVD) after CD31 staining of tumor sections of mice which were sacrificed 48 h after injection. Y-axes represents MVD at X 100 magnification. Three mice per group were included (n=3-5 areas per tumor).

was not optimal especially since the treatments had started at tumor size of 200 mm^3. This size could be too late for anti-angiogenic therapy because the tumors have already become well-vascularised which could make the anti-angiogenic effect insignificant. This is in line with reports in the literature, where most anti-angiogenic agents have been used against small size tumors [32], [33], [34]. However, it needs to be taken into account that in the clinic, most cancers are not detected at the early stage of tumor growth and therefore the clinical application of agents which would only be effective in small tumors could be questionable.

Another reason for the observed effects in our study could be the limited number of injections. Successful siRNA therapy has previously been achieved with 10 times of daily injection [34] which could not apply to our experiments since after the fifth injection the tumors reached the human endpoint (1500 mm^3) and the mice had to be sacrificed. So another advantage of starting therapy at a lower tumor size in further experiments could be that it would provide more time for a higher number of injections.

It could also be argued that silencing might have already occurred at a molecular level but the final impact on angiogenesis has not been sufficient to suppress tumor

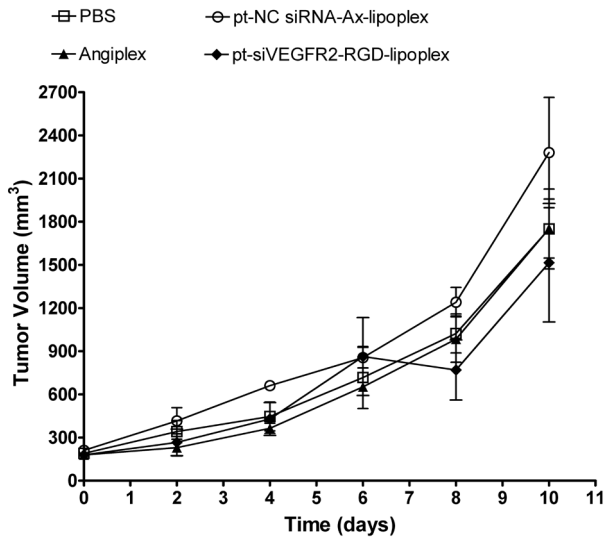

Figure 5 Anti-tumor efficacy of Angiplex after i.v. administration in A/J mice bearing Neuro2A tumors. Treatments consisted of Angiplex, pt-NC siRNA-Ax lipoplex, pt-siVEGFR2-RGD-lipoplex, and PBS. All treatments contained 15.2 nmol siRNA/ml. Results are presented as mean ± SD (n=3).

growth. To examine this hypothesis, we are planning to perform SL-RT PCR of RNA isolated from tumors to detect levels of siVEGFR2 and VEGFR2 mRNA as well as to do western-blot of tumor lysates to assess the amount of VEGFR2. These would provide us with information on whether the siVEGFR2 has reached the target and whether mRNA and VEGFR2 levels have been reduced compared to non-treated mice. In addition, as performed after single injection in the biodistribution study (section 3.4), microvessel density could be a more relevant indicator of anti-angiogenic effect which could be determined for the efficacy study as well after multiple injections. Since anti-angiogenic agents have a cytostatic role, using the same measurements as cytotoxic chemotherapeutics (such as tumor shrinkage) seem to be not a suitable tool to conclude their efficacy. Although in some clinical trials surrogate biomarkers such as microvessel density have been used to show efficacy, there is still a need for more direct biomarkers [35].

4. Conclusion

In this paper, we have presented a preliminary *in vivo* biodistribution and efficacy study of Angiplex (anginex-lipoplex containing siRNA against VEGFR2). This formulation is targeted to galectin-1 receptor on endothelial cells of tumor vasculature via the targeting peptide anginex. We performed the experiments in A/J mice bearing Neuro2A tumors and our results showed that siVEGFR2 could be detected in plasma of the mice treated with Angiplex until 48 h after i.v. injection while naked siVEGFR2 was degraded rapidly. This indicated that Angiplex maintained the siVEGFR2 intact which is necessary for delivery of the cargo to the target site. Analyzing the organs and the tumor revealed that siVEGFR2 could be recovered somewhat more in the lungs and spleen than the tumor or other organs. Detection of siVEGFR2 (and thus the particles) in the lungs could be due to interactions with galectin-1 receptors on the vast endothelium present in the lungs or simply because of the high amount of blood in the lungs as a result of their high vascularization. The latter seems more likely because no pulmonary adverse effects were observed in mice during the experiments which would indicate the absence of aggregation or interactions with lung capillaries. Recovering the particles from the spleen could demonstrate phagocytosis of particles. Therefore, in order to reach a better biodistribution profile we would suggest a higher PEG density. Angiplex was not present in high amounts in the tumor according to our studies which could be due to interactions of anginex with galectin-1 receptors on activated tissue macrophages or the secreted form of this protein. We would therefore suggest investigating other targeting ligands (such as RGD and nanobodies) in future experiments to compare with the results obtained for targeting with anginex. Moreover, to complete biodistribution studies, radio-labeled particles could be administered in further experiments to track down accumulation in organs and the tumor.

In the efficacy study, we found no significant tumor growth suppression in the group treated with Angiplex compared to the controls. This could firstly be because we started the treatment at a late stage of tumor growth while anti-angiogenic agents would show their effectiveness more on the neovasculature of smaller tumors. Secondly, it could be due to limited dosing which could be increased up to 10 injections in further experiments. Thirdly, tumor size might not be a suitable tool to show efficacy of anti-angiogenic agents and alternative methods evaluating microscopic effects on microvessels would be preferred. Finally, anti-angiogenic therapeutics are most beneficial in combination with chemotherapeutics [36], [37], [38]. Therefore it is recommended that for further research, treatment be started at a smaller tumor volume, the number of injections be increased to 10 times, more direct assays be used for evaluating anti-angiogenic effects, and Angiplex be used in combination with chemotherapeutics to achieve additive or synergistic effects which could plausibly be seen on the level of tumor growth inhibition.

5. Acknowledgements

This research was funded by the Dutch Top Institute Pharma project T3-301. We thank Dr. Roy van der Meel for helping with *in vivo* efficacy study and Joep van den Dikkenberg for cell culture assistance.

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7

Summary and discussion

Summary and discussion

In the battle with one of the most deadly diseases of our time, cancer, there is a high need for efficacious medicines. Novel nanomedicines represent a class of advanced drug delivery systems that can help increase the therapeutic index of anticancer agents. The use of conventional medicines to treat cancer is often hampered by their unfavorable safety profile which limits their dosing. By using targeted delivery systems, more accumulation at target tissues and/or less localization at sites sensitive to toxicity would be achieved. In addition, nanomedicines easily allow combination therapies by allowing the entrapment of more drugs in one nanoparticulate system. This can be beneficial to enforce synergistic or additive effects. An interesting therapeutic target in malignancies is the tumor neovasculature. After reaching a size of ~1-2 mm^3, tumors initiate angiogenesis, the process of formation of new blood vessels from pre-existing ones. Inhibiting the processes which are involved in angiogenesis are therefore valuable to stop access of tumor cells to nutrients and oxygen. Moreover, normalization of the tumor vasculature by low doses of anti-angiogenic therapeutics could potentially lead to better accessibility of chemotherapeutics to tumor cells [1], [2]. The main shortcoming of the current anti-angiogenic therapeutics is that they can exhibit severe adverse effects due to non-specificity [3], [4]. A promising alternative class of anti-angiogenic therapeutics currently under investigation is siRNA. siRNA can be rapidly designed to molecularly target the protein or proteins that are driving the angiogenic response. VEGF is a protein with a predominant role in promoting angiogenesis by binding to VEGFR-2 present on the endothelial cells of the tumor vasculature. This interaction results in the activation of certain signaling pathways which lead to pro-angiogenic events. Thus, silencing of VEGFR-2 expression by a specific siRNA could be an attractive approach in cancer therapy.

Systemic delivery of siRNA, however, has proven to be troublesome largely due to the complex environment that delivery systems encounter after administration. In **Chapter 2**, we have discussed extracellular and intracellular barriers that siRNA- and DNA-containing polymeric nanocarriers are confronted with *in vivo*. Although delivery of nucleic acids has shown impressive results *in vitro*, this can often not be translated to *in vivo* because of large differences in testing environments. We believe that by studying the environment that nanoparticles would pass *in vivo* on the way to the target site and the critical steps in functional performance, we can design *in vitro* assays reflecting the *in vivo* situation. Such relevant *in vitro* assays/strategies can then be used to design more efficient systems for systemic delivery. We have proposed a set of *in vitro* assays and strategies which could assess nanocarriers through these different steps before applying them *in vivo*. Thereby, the gap between the results originating from common *in vitro* tests (such as 2D-cell cultures and cytotoxicity

studies in static cell cultures) and those from *in vivo* experiments could become smaller which would directly decrease the extensive use of animals. Suggestions are:

1- Tests for predicting opsonization of nanocarriers by main serum proteins such as albumin, α2-macroglobulin, β2-glycoprotein I, apolipoprotein E, and fibronectin [5].
2- Size measurements of nanoparticles in buffer and serum/plasma by Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA) [6], flow cytometry [7], [8], and fluorescence Single Particle Tracking [9]. The data obtained by these techniques can help predict aggregation and dissociation of nanoparticles in the bloodstream.
3- Hemocompatibility assays of nanomedicines in blood by testing the potential of nanomedicines to affect hemolysis, thrombus formation, coagulation activation, and complement activation.
4- Cell culture experiments under simulated *in vivo* conditions. For instance, by using transwell systems in which nanoparticles can be added to the cells in a pulsatile fashion representing the dynamic blood flow (VI0.4 flow chamber: www.ibidi.de). Another example would be performing cell culture experiments in 3-D cultures rather than 2-D monolayer cultures in order to include cell-to-cell communication [10], [11], [12].
5- Studying viruses and nanocarriers which have entered clinical trials to implement their beneficial features in the design of novel carrier systems as has successfully been done in the case of multifunctional envelope-type nano devices which mimic an envelope virus [13].

In **Chapter 3**, we have investigated physical post-insertion of DSPE-PEG2000 into siRNA pHPMA-MPPM-based polyplexes. Although PEGylation is a common method for reducing the surface charge of nanoparticles and delaying their clearance [14], its application is not very successful yet in the case of nucleic acid delivery systems and also controversial as recently described as the PEG dilemma [15]. It is assumed that long exposure to PEG in the circulation would stimulate production of anti-PEG IgM [16]. In addition, PEGylated particles being hydrophilic and having a (close to) neutral charge likely interact less with target cell. PEG has also been reported to delay endosomal escape which is an important limiting step in intracellular nucleic acid delivery [17]. Therefore, 'temporary' PEGylation seems a suitable alternative. It has previously been found that physical post-insertion of DSPE-PEG into liposomes, lipid nanocapsules, and liposome-polycation-DNA complexes, leads to better escape from the mononuclear phagocyte system due to the shielding effect of PEG molecules [18], [19], [20]. In addition, since the inserted PEG is not covalently bound, it could dissociate relatively easily overcoming the limitations that a covalently-bound PEG would impose on endosomal escape. We have exercised this concept for a polymer-based nanocarrier. This carrier accumulates mainly in the spleen after

i.p. administration in mice due to its positive surface charge [21]. After the post-insertion procedure, we observed a drop of the surface charge of the polyplexes at different N/Ps alongside the facilitation of siRNA release from the polyplexes. The latter observation could indicate a decreased siRNA retention within the formulation. Besides, post-insertion of DSPE-PEG2000 reduced the tightness of the siRNA/pHPMA-MPPM complexes and decreased cellular toxicity. It is known that in aqueous dispersions, amphiphiles can transfer from micelles to phospholipid vesicles at a certain hydrophilic-hydrophobic balance [22]. Our findings indicated that post-insertion occurred plausibly through hydrophobic interactions of the phospholipid tail of DSPE-PEG2000 with the hydrophobic backbone of the polymer leading to an equilibrium mixture of micelles and polyplex-absorbed DSPE-PEG2000. These results seem encouraging to be pursued as a 'temporary' means of adding PEG to already established formulations. However, the exact mechanism of interactions remains to be elucidated by performing comparative studies with other polymers with different hydrophobicities of the backbone. In addition, the biological activity of polyplexes based on these polymers can reveal the impact of post-insertion on endosomal escape.

We have introduced a 'dual action' targeted lipoplex system (Angiplex) containing siVEGFR-2 as an anti-angiogenic therapeutic in **Chapter 4**. This formulation includes an siRNA-protamine core with a positive charge which is complexed with liposomes composed of DOPE, CHEMS, and DSPE-PEG2000, DSPE-PEG2000-maleimide. DOPE was included because it is a fusogenic lipid which changes from lamellar to hexagonal phase upon acidification thus creating a negative curvature which facilitates endosomal escape. DSPE-PEG2000 was used to create a stealth effect and DSPE-PEG2000-maleimide was included to allow coupling of the targeting peptide (anginex) to the surface of the lipoplexes. Anginex is a 33-mer peptide targeting galectin-1 receptor which is overexpressed on endothelial cells of tumor vasculature [23], [24]. The overall surface charge of Angiplex is slightly negative. Although cationic delivery vehicles complex strongly with siRNA via charge interactions, their stability can be compromised because of interaction with blood components [25], [26]. Therefore, we continued to explore the possibility of using Angiplex as a slightly negatively-charged particle for silencing the expression of VEGFR-2 gene in human umbilical vein endothelial cells (HUVECs). Angiplex showed low toxicity for HUVECs and was internalized specifically as demonstrated in competition assay studies in Chapter 4. Cellular uptake of Angiplex was significantly higher than a control formulation which contained a negative control peptide (b-peptide-28). Gene silencing experiments revealed a 61% silencing efficiency for Angiplex. This effect could be a consequence of RNAi by siVEGFR-2 and crosstalk between galectin-1 and neuropilin. Because it is known that binding of anginex peptide to galectin-1 would activate this receptor and lead to crosstalk between

galectin-1 and neuropilin. This crosstalk results in the down-regulation of the VEGFR-2-mediated signaling pathway [27]. This dual action approach appears promising because the strategy influences the target protein expression from two different angles. The benefits of multiple-targeting of different pathways has been previously reported in the literature [28], [29], [30], [31]. For instance, delivery of siRNA against signal transducer and activator of transcription-3 by a B-cell-activating factor (BAFF)-receptor aptamer has been reported to have a double inhibitory effect on B-cell proliferation firstly through BAFF-receptor interaction with the aptamer and secondly through RNAi [32]. In another study, van der Meel *et al*. have found that the use of liposomes containing AG538 decorated with EGa1 nanobodies induces a higher antitumor efficacy than empty EGa1-liposomes or bare AG538 liposomes. They have related this observation to inhibition of the EGFR pathway by the nanobody on the one hand, and to the IGF-1R inhibition by AG538 on the other hand [33].

Hemocompatibility of nanocarriers is a major concern in case of parenteral administration, in particular after intravenous administration. Therefore, we studied Angiplex and pHPMA-MPPM-based polyplexes for hemocompatibility parameters in **Chapter 5**. Since platelets play an important role in thrombosis (formation of blood clots within the vessels) related to foreign particles firstly platelet activation and aggregation were evaluated. Two main marker proteins for platelet activation are P-selectin and fibrinogen. The former is an adhesive membrane protein which transfers to the platelet surface after vascular injury in order to assist with platelet accumulation [34]. The latter is released from secretion granules of platelets and is involved in creating bridges between platelets to promote aggregation [35]. The presence of these markers on platelets was significantly lower after incubation with Angiplex compared to pHPMA-MPPM polyplex likely due to the higher hydrophilicity of Angiplex by the presence of PEG molecules. Particles with a hydrophobic surface have been shown to trigger platelet activation [36]. The observed high activation by pHPMA-MPPM–based polyplexes could be related to their cationic nature which would lead to binding to platelets via charge interactions [37]. These experiments were performed both with isolated platelets and in whole blood and activation was always higher in the former. The reasons could be that platelets became pre-activated during the additional steps needed for their isolation [38]. Alternatively, in whole blood, cationic particles may have become opsonized with blood proteins before interacting with platelets. This cover of endogenous proteins may have reduced platelet activation in whole blood. Subsequent aggregation studies confirmed the above findings as platelet aggregation caused by pHPMA-MPPM polyplex was two times higher than that of Angiplex.

Stimulation of the intrinsic pathway of coagulation was tested by contact system activation studies which revealed a low effect of both formulations on this pathway. It is known that the contact system is particularly activated by negatively charged [39] and hydrophobic particles [40] which would explain our results.

Another issue with systemically administered nanocarriers is immune response stimulation. One aspect of this response is TLR activation. The TLRs constitute a class of transmembrane proteins which are mainly expressed on antigen presenting cells (APCs) and recognize microorganism-associated molecular patterns. Activation of APCs by TLR signaling would lead to expression of costimulatory molecules such as CD40 and CD86 and consequently immune stimulation. According to our studies, Angiplex did not induce a significant activation of TLR2, 3, 4, or 9. Also, Angiplex did not cause overexpression of CD40, CD86, or MHC II. These observations are likely due to the shielding of surface charge by PEG which would make the particles less recognizable by immune cells.

The complement system also plays a key role in host defense. Therefore, we evaluated the formation of complement factor SC5b-9 after incubating human sera with Angiplex and pHPMA-MPPM polyplex. Our results indicated that both formulations did not activate the complement system considerably. However, the slightly higher activation due to Angiplex compared to pHPMA-MPPM polyplex could be due to the negative charge of phosphate moieties in the structure of phospholipids [41]. Moreover, it was found that free maleimide groups on Angiplex which had remained after coupling of the peptide, increased complement activation. Thus, for further applications of this formulation it is suggested to quench maleimide groups with b-mercaptoethanol [42]. In spite of the fact that literature reports have shown that positively charged particles tend to activate the complement system, which would make activation likely for pHPMA-MPPM, it has also been reported that complexation with nucleic acids decreases the accessible positive charges and therefore leads to reduced activation [43]. Likely, this was seen in our experiments as well.

Encouraged by the promising *in vitro* results of Angiplex, we proceeded to an *in vivo* biodistribution and efficacy study in a Neuro-2A tumor model in A/J mice as described in **Chapter 6**. We found that Angiplex could maintain siVEGFR2 intact as siVEGFR2 could be detected in plasma by SL-RT PCR up to 48 h after i.v. injection. However, it did not lead to significant tumor growth inhibition. Protecting the siRNA from degradation in the blood is a crucial property of a successful delivery system because any degradation would result in loss of functionality. Analyzing the organs and the tumor revealed that Angiplex accumulated more in the lungs and spleen than in the tumor or other organs which could explain lack of efficacy. Plausibly, this is due to the lungs containing a huge number of vessels and thus a high amount of blood. It seems unlikely that the above finding is due to entrapment of particles in the lung capillaries because no pulmonary adverse effects occurred during the experiments. Detecting siVEGFR2 in the spleen could be a result of phagocytosis which could be circumvented by using a higher PEG density. Concluding from our *in vitro* and *in vivo* observations and given the fact that galectin-1 is expressed in tissue macrophages and it also exists in an extracellular form, we could argue that another receptor more

specific to tumor endothelial cells would be a better choice for targeting to the tumor vasculature. In addition, for further experiments we would recommend an increase in the number of injections, starting the injections when tumors are less-vascularized (smaller), and combination therapy with chemotherapeutics because it has been reported to lead to additive or synergistic effects [1], [2], [44]. Finally, anti-angiogenic effect is suggested to be investigated on the molecular level (by evaluating the mRNA or protein expression) to provide direct evidence of biological activity *in vivo*.

Conclusion and future perspectives

In this thesis, we have studied a lipid-based targeted nanoparticle system (Angiplex) for delivery of siVEGFR-2 to tumor endothelial cells *in vitro* and *in vivo*. We found that Angiplex was not toxic towards HUVECs *in vitro* and showed considerable gene silencing activity. The mechanism of cellular internalization is hypothesized to be through interactions of anginex peptide of the lipoplexes with galectin-1 receptors on endothelial cells. It would be interesting in the future to track these nanoparticles in the cells by fluorescence microscopy methods in order to determine their uptake route as well as their localization in intracellular compartments and their unpacking.

In vivo, this formulation was well-tolerated in mice and circulated in the blood for 48 hours. However, Angiplex accumulated more in the lung and spleen than the tumor and other organs and showed low efficacy. We would recommend modifications in the set-up of the efficacy study so that the treatments start at a smaller tumor size to be able to reach neovasculature alongside increasing the number of injections. We would also suggest using a higher density of PEG in the formulations to reduce phagocytosis and comparing the results obtained here with other targeting ligands to a more specific receptor in the endothelial cells of the tumor vasculature. Also, it would be expected that investigating changes in mRNA levels or expression of VEGFR2 would provide more direct evidence on the efficacy of the delivery system at this stage.

Another topic explored in this thesis was hemocompatibility of Angiplex compared to pHPMA-MPPM polyplex. A correlation between physicochemical characteristics of these formulations and their interactions with platelets and the complement system was found. We showed that Angiplex, being negatively charged, could be advantageous over pHPMA-MPPM polyplex, being positively charged, because of having marginal effects on platelet activation and aggregation. Complement activation by both formulations was low. We would suggest the performance of hemocompatibility assays presented here as a preliminary step before administering particles *in vivo* to predict their behavior which would help limit animal usage. In an effort to decrease interactions of pHPMA-MPPM polyplexes with

blood components, we investigated a simple PEGylation method based on post-insertion of PEG phospholipids. This method is interesting because it provides the possibility of temporary PEGylation which could prevent adverse effects caused by PEG. Despite the promising results obtained, the exact mechanism of post insertion still needs to be revealed by performing functional assays with such polyplexes in comparison with other systems with different physicochemical properties.

It should be taken into account that application of siRNA has not been without side effects. There have been reports of saturation of proteins involved in the RNAi process by introduction of exogenous siRNA which could be alleviated by administering the minimum therapeutic dose. Also, it has been discovered that siRNA can bind non-specifically to non-target transcripts which would create off-target effects. Strategies can be used to minimize these effects such as 2´-O-methylation of siRNA molecules.

In conclusion, the Angiplex lipoplex system developed in this thesis seems to have enough promising properties justifying further research towards *in vivo* studies. Moreover, the *in vitro* assays and strategies presented in this thesis to evaluate the hemocompatibility of nanoparticles under conditions relevant for the *in vivo* situation, can be recommended as standard screening tests to limit the number of experimental animals in preclinical research.

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Appendices

Nederlandse samenvatting

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Nederlandse samenvatting

De groei van solide tumoren is afhankelijk van de aanvoer van zuurstof en voedingsstoffen via het bloed. Hiervoor scheidt de groeiende tumor een hoeveelheid groeifactoren uit die de aanmaak van nieuwe bloedvaten naar en in het tumorweefsel bevordert. Dit proces wordt ook wel angiogenese genoemd. Door receptoren op het oppervlak van endotheelcellen die betrokken zijn bij de angiogenese te blokkeren, kan de tumor in zijn groei geremd worden. Eén van die receptoren is de vasculair endotheliale groeifactor receptor-2 (VEGFR-2).

In dit onderzoek is getracht VEGFR-2 expressie op het tumor vaatbed te inhiberen met behulp van RNA interferentie (RNAi). Hiervoor moeten kleine stukjes dubbelstrengs RNA, ook wel siRNA genoemd, in de cel gebracht worden waarvoor vaak nanodeeltjes gebruikt worden die het siRNA beschermen tegen afbraak in de bloedbaan en het intact afleveren in het cytosol van de endotheelcellen in de tumor.

De gerichte afgifte van siRNA met nanocarriers is echter lastig mede door de complexiteit van de omgeving waarin deze deeltjes na intraveneuze toediening terecht komen. In **hoofdstuk 2** wordt een overzicht gegeven van de intracellulaire en extracellulaire barrières die siRNA of DNA bevattende polymere nanodeeltjes in het lichaam moeten beslechten. Tevens wordt een aantal strategieën besproken die toegepast zijn om zulke barrières te overwinnen. Hoewel de afgifte van nucleïnezuren met zulke polymere nanodeeltjes goede resultaten heeft opgeleverd *in vitro* zijn de resultaten *in vivo* vaak teleurstellend. Er worden suggesties gegeven hoe de kloof tussen de *in vitro* en *in vivo* testcondities kleiner gemaakt kan worden door geavanceerde *in vitro* modellen toe te passen in plaats van de huidige *in vitro* testcondities gebaseerd op eenvoudige 2D kweken van goed te transfecteren cellen. De verwachting is dat met het implementeren van zulke geavanceerde *in vitro* modellen het proefdiergebruik omlaag kan en er vooraf een betere selectie gemaakt kan worden van dragersystemen die *in vivo* effectief zullen zijn.

In **hoofdstuk 3** wordt de post-insertie van DSPE-PEG2000 in siRNA-pHPMA-MPPM polyplexen beschreven als een makkelijke methode om de oppervlaktelading van deze cationische deeltjes (tijdelijk) af te schermen. PEGylering is een veel gebruikte techniek om de pharmacokinetiek van peptiden, eiwitten en nanodeeltjes gunstig te beinvloeden door de klaring van deze macromoleculen en deeltjes tegen te gaan. Het heeft echter ook een nadelig effect op het vrijkomen van de therapeutische lading uit nanodeeltjes, wat ook wel het “PEG dilemma” wordt genoemd. Een oplossing is tijdelijke PEGylering zodat het nanodeeltje voldoende lang circuleert, maar eenmaal op de plek van bestemming in het lichaam, zijn PEG laag verliest om zo celopname en cargo afgifte mogelijk te maken. De strategie voor tijdelijke associatie van PEG met polyplexen die hier is uitgetest is het inbouwen van PEG lipiden na vorming van de polyplexen door PEG-lipide micellen toe te voegen aan de

polyplexen. De resultaten tonen aan dat DSPE-PEG2000 inderdaad een interactie aangaat met de cationische polyplexen waarbij de Z-potentiaal van de deeltjes afneemt en de interactie tussen polymeer en siRNA minder sterk wordt. Hoewel verder onderzoek naar de stabiliteit van deze deeltjes noodzakelijk is, geven deze eerste resultaten aan dat post-insertie van PEG-lipiden een serieuze optie is om de ongewenste positieve lading van polyplexen tijdelijk af te schermen hetgeen noodzakelijk is voor systemische toepassingen.

Hoofdstuk 4 beschrijft de ontwikkeling van Angiplex, een siRNA bevattend nanodeeltje bestaande uit een protamine/siRNA kern en een PEG-lipide buitenlaag die met behulp van een peptide (anginex) getarget wordt naar de tumor vasculatuur. Angiplex heeft een dubbele werking daar het angiogenese kan remmen door te binden aan galectin-1 maar ook door de intracellulaire afgifte van siVEGFR-2. De werking van Angiplex is getest op humane navelstreng endotheelcellen (HUVECs). De resultaten laten zien dat Angiplex specifiek bindt aan en opgenomen wordt door HUVECs. Afgifte van siVEGFR-2 met behulp van Angiplex leidde tot 61% reductie in genexpressie van VEGFR-2 die mogelijk veroorzaakt wordt door een direct effect van RNAi in combinatie met galectin-1 binding.

Voor frequente, parenterale toediening van nanodeeltjes voor therapeutische doeleinden is hemocompatibiliteit essentieel. In **hoofdstuk 5** is daarom de hemocompatibiliteit van Angiplex en pHPMA-MPPM polyplexen bestudeerd met een set van *in vitro* assays, waaronder bloedplaatjes-, complement- en Toll-like receptor- (TLR) activatietesten. Het onderzoek toont aan dat incubatie van bloedplaatjes met Angiplex formuleringen niet leidt tot plaatjesactivatie, terwijl pHPMA-MPPM polyplexen leidde tot sterke activatie en aggregatie van bloedplaatjes, waarschijnlijk door de positieve lading van deze deeltjes. Beide formuleringen lieten een minimale activatie van het complementsysteem zien en resulteerde ook niet in activatie van de stollingscascade via de intrinsieke keten. Deze hemocompatibiliteitsassays stellen ons in staat een gedegen onderzoek te doen naar mogelijk ongewenste interacties van nanodeeltjes met componenten in het bloed voordat deze systemen *in vivo* getest gaan worden.

Hoofdstuk 6 beschrijft de biodistributie en therapeutische effectiviteit van Angiplex in een neuroblastoma (Neuro2a) tumor model in muizen. Intraveneuze injectie van Angiplex leidde tot verlengde bloedwaardetijden van intact siVEGFR-2 in vergelijking met toediening van vrij siVEGFR-2. Dit leidde in deze muizen echter niet tot een significante inhibitie van de tumorgroei. Analyse van de hoeveelheden intact siVEGFR-2 in de verschillende organen laat zien dat met Angiplex het siRNA voornamelijk in de longen en milt te terug te vinden is en niet zozeer in het tumorweefsel wat mogelijk een verklaring is voor de afwezigheid van een therapeutisch effect.

Acknowledgements

What an adventure it has been doing this Ph.D. project! But of course, the work done during the past four years would not have been possible without the support of many people inside or outside the lab.

Firstly, I would like to thank you Gert, dear promotor, for your fruitful input, for not letting the project fall off the main path, and for creating collaborations. Somehow, you always found the time in the middle of your busy schedule!

Dear Wim, thank you for giving me the opportunity to join your group first as a guest researcher and then as a Ph.D. candidate. On my journey in Biofarmacie, I learnt a lot from your management skills and in parallel I appreciated your sense of humor!

I would like to thank my co-promotor and daily supervisor Enrico, who supported me immensely during these four years of Ph.D. Dear Enrico, thank you for being very much involved in the project with a critical (but also quite positive) eye and for teaching me how to think analytically and work independently. You helped me stay positive even in the darkest of times!

Dear Ray, my second co-promotor, I really appreciated your super-positive attitude that made me believe there is always a way and an impasse doesn't exist! ☺ I would also like to thank you for your scientific feedback during the project.

My sincere appreciation goes next to InteRNA and VUMC collaborators: Dear Paula, dear Roel, many thanks to you for your effective input during the meetings. Dear Arjan, dear Judy, thank you for your useful feedback and discussions during the project meetings.

Dear Robbert Jan, thank you for the scientific discussions we had about the complement activation project.

From "just colleagues" to "great friends", that's how I see my encounter with you, dear Maryam! You always inspired and supported me with your strong character, positive attitude, and great advice. I had such fantastic times with you also outside the lab while going to concerts, watching movies, or going to parties plus awesome memories of all the fun we had in Lanzarote! ☺

Another person who guided me through the maze of Ph.D. was dear Meriem. Thank you very much for being so open to questions and your practical approach towards how things should come out in the end.

Dear Roy, thank you for all the guidance in the lab and your patience when I always had questions! And thanks for the detailed ski-holiday instructions in Austria! Dear Erik O, thanks for your commitment during the in vivo study! Dear Ebel, during the animal experiments you were most helpful with your skills and your calm attitude. Thank you! Dear Joep, thank you for your advice in the cell culture lab and for managing the HUVECs crisis! ☺ Looking back at the time spent in the lab reminds

me of the great help that Mies always offers to everyone among whom I'm no exception. Dankje wel Mies! Thank you Louis and Georgi for your support in the lab.

Dear Barbara, I really appreciated our chats, your good humor, and your help. ☺

And of course the "UMC lunch group" (Markus, Roy, Sytze, Meriem, Niels, Kristel, Jos, Isil (the time keeper!), Erik O, Jan Jaap): it was great to have those 12.10 (sharp!) lunches together mixed with relaxed conversations with you.

Dear Kimberley, I will miss all those sympathizing conversations we had as neighbors and as Ph.D. students-approaching-the-finish-line! To my office-area mates: Neda S, Neda K, Louann, Mohadeseh, Yinan, and Dandan: Thank you for the nice atmosphere! Dear Yang, Luis, Isil: great times in the CRS! Dear Andhyk, thanks for your help in the lab. Dear Mazda, thank you for your scientific suggestions and discussions.

And a word of gratitude to my other/former Biofarmacie colleagues: Erik T, Merel, Burcin, Reka, Emmy, Melody (I appreciate your helpful attitude and your energy! All those great times we had at your colorful Christmas parties, at movie festivals, and at ladies' nights!), Inge-Mike (Sometimes eating burgers all-day-long with you guys and sometimes watching creepy movies at fantasy film festivals! I liked it all!), Anastasia (I really enjoyed the time we spent together during your stay in Utrecht!), Laura, Hajar, Pieter, Bart, and Joris.

Dear Nahid, dear Soheil, thank you very much for your help!

Dear Mark, Rick, and Thijs from InteRNA: thanks for performing the qPCRs en bedankt voor de gezelligheid!

During my Ph.D. I had the privilege to supervise some brilliant students: dear Carmen, dear Paola, dear Marianne; I would like to thank you all for your enthusiasm and the energy that you devoted to your projects.

Being able to succeed in my Ph.D. included not only the ones involved scientifically in the project but also all those people who know very well how to give you support even though they might not know how an siRNA delivery system should work. ☺ I would like to thank those people here:

Lieve Muriel, zeer bedankt! For all those lively chats over dinner, for your sound advice and valuable support when things looked gloomy, for your natural openness, and for the lovely times we had during concerts and performances. Lieve Lisette, I always have such a great time with you whether it is just sipping a cappuccino in the "Village", practicing Dutch, watching a dance performance, or partying for the oud-en-nieuw! Thank you for being there when things were going wrong and for transferring your positive cheerful attitude to me! Dear Scott, how great it has been to get to know you since I moved to the Netherlands! Without your constant optimism I might have not got over some of the obstacles on my way during the past few years. I hope we keep on having our philosophical discussions, watching art spectacles, and ontdekk-ing gezellige spots of Utrecht! Dear Debbie, chica! I really appreciated

our human-behavior discussions, our lovely dinners, and our brilliant trip to Barcelona! Dear Kami, I'm grateful to your wisdom, your understanding, and your special way of making things easy. Dear Farnaz, you were a great source of inspiration for me when I started my Ph.D. Thank you for your advice and your joyful attitude! Dear Shabnam, thank you for all the wonderful times in Tehran; sometimes chatting, sometimes training, and sometimes thinking together!

I would like to thank my wonderful parents for their unending love and support from close-by or far-away: **مامان عزیزم**, thank you very much for always encouraging me to leap further and even further! Your strong character has always inspired me. **بابای عزیزم**, thank you very much for your wise advice and great support.

My wonderful brother, **اردلان جان**, your limitless caring attitude amazes me again and again! Thank you for our spirited discussions, your honest realistic opinions, and for the great times we have every time I come to the other side of the planet! ☺

Dewi **جان**, my love, you were always there supporting me during the hardest part of my Ph.D. with a warm smile and incredible calmness even when the sky was **خاکستری**! ☺ I'm very happy to get to know you more and more every day and to experience my life further beside you.

Curriculum vitae

Afrouz Yousefi was born on the 19th of September 1982 in Tehran, Iran. After finishing high school, she participated in the National University Entrance Exam for Medical Sciences where she was accepted to study Pharmacy at faculty of Pharmacy of Tehran University of Medical Sciences. She obtained her Pharmacy Doctorate degree (PharmD.) in 2008 after defending her thesis entitled “Evaluation of side-chain liquid crystal polymers in thermoresponsive drug delivery systems”. She performed this research under the supervision of Prof. dr. R. Dinarvand. During her studies she participated in an Industrial Pharmacy internship program for 5 months at the Jaber-Ebne-Hayyan Pharmaceutical Company in Tehran. In the last year of her studies she worked as a scientist at the R&D department of Sobhan Oncology, Co. in Tehran working on the formulation of Docetaxel liposomes. In 2009, she started working as a guest researcher at the department of Pharmaceutics, Utrecht University, under the supervision of Prof. dr. G. Storm for 6 months. During this period, she investigated binding of liposomes to plasma proteins by a Surface Plasmon Resonance-based method. Subsequently, she started her Ph.D. project in late 2009 at the Utrecht Institute for Pharmaceutical Sciences, department of Pharmaceutics, Utrecht University under the supervision of Prof. dr. G. Storm, dr. E. Mastrobattista, and dr. R.M. Schiffelers. The results of her work are presented in this thesis.

List of publications

Publications from this thesis:

Afrouz Yousefi, Gert Storm, Raymond M. Schiffelers, Enrico Mastrobattista. Trends in polymeric delivery of nucleic acids to tumors. J. Control. Rel. 170(2):209-218 (2013)

Afrouz Yousefi, Meriem Bourajjaj, Negar Babae, Paula I. van Noort, Roel Q. J. Schaapveld, Judy R. van Beijnum, Arjan W. Griffioen, Gert Storm, Raymond M. Schiffelers, Enrico Mastrobattista. Dual action lipoplexes for delivery of anti-angiogenic siRNA. (Submitted for publication)

Afrouz Yousefi, Marianne Lauwers, A. Marit de Groot, Reka Nemes, Thijs van Holten, Negar Babae, Alice J. A. M. Sijts, Mark Roest, Gert Storm, Raymond M. Schiffelers, Enrico Mastrobattista. A comparison of hemocompatibility between a lipid-based and a polymer-based siRNA nanocarrier formulation. (Submitted for publication)

Negar Babae, Meriem Bourajjaj, Yijia Liu, Judy R. van Beijnum, Francesco Cerisoli, Puthupparampil V. Scaria, Mark Verheul, Maaike P. van Berkel, Ebel H.E. Pieters, Afrouz Yousefi, Enrico Mastrobattista, Gert Storm, Eugene Berezikov, Edwin Cuppen, Martin Woodle, Roel Q. J. Schaapveld, Gregoire P. Prevost, Arjan W. Griffioen, Paula I. van Noort, Raymond M. Schiffelers. Systemic miRNA-7 delivery inhibits tumor angiogenesis and growth in murine xenograft glioblastoma. (Submitted for publication)

Negar Babae, Meriem Bourajjaj, Mark Verheul, Judy R. van Beijnum, Rajshri N. Lalai, Francesco Cerisoli, Afrouz Yousefi, Enrico Mastrobattista, Gert Storm, Edwin Cuppen, Roel Q. J. Schaapveld, Arjan W. Griffioen, Paula I. van Noort, Raymond M. Schiffelers. MicroRNA-142-3p inhibits angiogenic endothelial cells via integrin alpha V and RAC1. (Ready for submission)

Negar Babae, Meriem Bourajjaj, Yijia Liu, Rick J van Haastert, Puthupparampil V. Scaria, Maaike P. van Berkel, Ebel H.E. Pieters, Judy R. van Beijnum, Afrouz Yousefi, Enrico Mastrobattista, Gert Storm, Edwin Cuppen, Martin Woodle, Roel Q. J. Schaapveld, Gregoire P. Prevost, Arjan W. Griffioen, Paula I. van Noort, Raymond M. Schiffelers. Targeted delivery of miR-9* has anti-angiogenic activity in a mouse tumor model. (In preparation)

Other publications:

Bart Crielaard†, Afrouz Yousefi†, Joris Schillemans, Twan Lammers, Gert Storm. Interaction of liposomes with proteins measured by surface plasmon resonance correlates with their *in vivo* circulation time. J. Control. Rel. 148(1): e32-3 (2010)

Selected Abstracts:

Afrouz Yousefi, Roy van der Meel, Negar Babae, Meriem Bourajjaj, Judy R. van Beijnum, Arjan W. Griffioen, Paula I. van Noort, Roel Q. J. Schaapveld, Gert Storm, Raymond M. Schiffelers, Enrico Mastrobattista. *In vitro* characterization of a lipidic delivery system for anti-angiogenic siRNA. Annual Meeting and Exposition of the Controlled Release Society, Honolulu (2013; poster presentation)

Afrouz Yousefi, Roy van der Meel, Negar Babae, Meriem Bourajjaj, Judy R. van Beijnum, Arjan W. Griffioen, Roel Q. J. Schaapveld, Paula I. van Noort, Gert Storm, Raymond M. Schiffelers, Enrico Mastrobattista. *In vitro* characterization of a lipidic delivery system for anti-angiogenic siRNA. FIGON Dutch Medicine Days, Lunteren (2012; poster presentation)

† These authors contributed equally to the work.