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Abstract

Over the last decade, considerable effort has been put in the implementation of RNA interference (RNAi) as a treatment for various disorders. As RNAi occurs in the cytoplasm of cells, it is imperative that RNAi mediators such as small interfering RNA (siRNA) cross several extracellular and intracellular barriers to reach this site of action. Among the extensive range of proposed delivery systems for siRNA, matrix systems possess interesting properties to promote the delivery of siRNA to a target tissue. In this review, a number of recently developed matrix and hybrid systems for siRNA delivery are discussed.

Introduction

With the discovery of the RNAi mechanism in *C. elegans* and the elucidation of a similar mechanism in human cells [1, 2] a mechanism for a novel therapeutic strategy was found. The possibility to target almost every gene transcript with unprecedented specificity and effects lasting for several days contribute to the promising prospects of RNAi for therapeutic use [3]. Moreover, with RNAi, researchers now have access to potentially interesting targets emerging from genomic data, that cannot readily be targeted with conventional small molecule pharmaceuticals. However, RNAi mediators such as siRNA possess a high negative charge (~40 per siRNA duplex) and high molecular weight (~14-18 kDa) implying that these molecules do not possess optimal drug-like properties and that classic pharmaceutical formulations will not suffice for the application of RNAi *in vivo*. Despite these unfavorable properties, siRNA needs to overcome multiple barriers, both extra- and intracellular, before it can interact with the RISC complex in the cytoplasm of a target cell and perform its designated function [4, 5]. On the extracellular level, degradation by serum nucleases and fast renal clearance limit the circulation time of naked siRNA upon injection in the blood stream to a few minutes, which is far too short to achieve adequate biodistribution of the siRNA [6-8]. As a consequence, the efficacy of the siRNA will be seriously limited when extravasation of the siRNA to the interstitial fluids is required in order to reach the target cells.

For this reason, a great deal of effort has been put in the development of carrier systems for delivering siRNA to the cytoplasm of the target cells. Incorporation of the siRNA into (nano)particulate delivery carriers can not only influence the biodistribution of the siRNA by its protection against nuclease degradation and fast glomerular filtration, it can also improve the actual delivery of the siRNA to the cytoplasm of the target cells [9, 10] (Figure 1). Although naked siRNA is not readily taken up by target cells, its internalization can be accomplished via the endocytosis of delivery systems containing siRNA. However, endocytosis results in the accumulation of the

incorporated siRNA in the endosomal compartments, from which escape is considered an important intracellular barrier further limiting the amount of siRNA delivered to the cytosol [5, 11, 12]. For this reason, many authors have focused on strategies which either stimulate endosomal escape or avoid the endosomal pathway [13-18].

An important strategy to improve the prospects of siRNA based therapy is the development of carefully designed siRNA duplexes which show marked advantages compared to non-modified siRNA duplexes. Kim et al. discovered that synthetic RNA duplexes 25-30 nucleotides in length can be up to 100-fold more potent than corresponding conventional 21-mer siRNAs because they are substrates of the Dicer endonuclease, directly linking the production of siRNA to the incorporation in the RNA-induced silencing complex (RISC) [19]. In addition, Rose and colleagues discovered that the 2-nucleotide 3'-overhang is the predominant determinant of which strand participates in the RNAi pathway. By using asymmetric siRNA, the specific loading of the desired siRNA strand can be achieved which translates into a markedly improved silencing efficiency [20-24]. Additionally, some important chemical modifications are incorporation of locked nucleic acids (LNAs), DNA residues or 2' O-methyl ribonucleosides [25, 26]. In summary, judicious chemical modification of siRNA duplexes can result in increased gene silencing efficiency and decreased susceptibility to serum nucleases, off-target silencing and immune responses [3, 8]. Although the benefits of chemically modified siRNA are unquestioned, these modifications alone will not solve all problems regarding siRNA delivery, mainly because they will not aid in guiding the siRNA to the cytoplasm of the desired target cells. For this reason, modifications of siRNA duplexes are often used in combination with other strategies such as bioconjugation, complexation or encapsulation.

siRNA has been chemically conjugated to a variety of bioactive molecules, lipids, polymers, peptides and inorganic nanostructured materials to enhance their pharmacokinetic behavior, cellular uptake, target specificity and safety. Most conjugates employ acid-labile and reducible linkages between the siRNA and the conjugation compound so unconjugated siRNA can be released upon exposure to the acidic endosomal environment and the reductive milieu of the cytosol respectively. An alternative strategy is to make use of endogenous Dicer, which can process a double stranded RNA conjugate to generate siRNA [27, 28]. An overview of siRNA conjugate delivery systems and some promising *in vivo* results can be found in an excellent review by Jeong et al. [29].

Introduction of nucleic acids into target cells is a naturally occurring process since viruses have evolved for millions of years to effectively deliver genetic material to host cells. In an attempt to mimic these functions, a variety of viral vectors has been employed to deliver custom genetic material to cells to provide either transient (i.e. adenovirus, vaccinia virus) or permanent (i.e. retrovirus, adeno-associated virus) transgene expression in target cells [30]. Although virus-mediated delivery methods for RNAi are usually based on delivery of genes encoding for small hairpin RNA (shRNA), viral vectors are also being used to deliver siRNA *in vivo* [30]. Despite being a very effective delivery system, the safety of viral delivery systems is still questionable as control over the transduced cell type, inflammatory responses, immunogenicity and oncogenic transformations are still considered serious problems in the exploitation of viral delivery systems [31-33]. For this reason, especially siRNA delivery via non-viral delivery systems is currently under intensive investigation.

In contrast to viral carriers, non viral delivery systems such as polyplexes, lipoplexes, vesicular or matrix systems have been proposed as a safer alternative for siRNA delivery. Polyplexes are formed by complexation of negatively charged nucleic acids with positively charged polymers and are reviewed elsewhere (Ref to siRNA issue – Polyplexes). Similarly, complexation of nucleic acids with cationic lipids result in lipoplexes and are considered to be the golden standard for *in vitro* transfections (Ref to siRNA issue). Vesicular systems are defined as colloidal systems where the active compound is trapped inside an oil or water cavity surrounded by a membrane made of polymers, surfactants or lipids [34] while matrix systems, being the focus of this review, are three-dimensional networks that are formed by physical or chemical crosslinking of polymers. An important distinction from numerous other delivery systems is that the active compound is structurally not a part of the matrix itself. Many of these matrices are designated as hydrogels, which are by definition hydrophilic polymeric networks with a three-dimensional configuration capable of imbibing high amounts of water or biological fluids [34-37]. However, some important and highly investigated matrix systems, i.e. those based on poly(lactic acid) (PLA) or poly(D,L-lactide-co-glycolide) (PLGA), can only absorb a limited amount of water (< 5-10%) and are consequently not classified as hydrogels [36].

Matrix systems are known for their enhanced physicochemical stability and biocompatibility as most of their constituents are often derived from naturally occurring biopolymers. Their versatility is emphasized by reports on matrix surface modification, incorporation of stimuli responsive elements or controlled release of the active

compound from the delivery system through its slow diffusion or surface erosion. To our knowledge this is the first review on matrix based siRNA delivery systems.

1. siRNA delivery via matrix systems

Since RNAi can generally be applied to interfere with the expression of virtually any gene, its therapeutic potential is practically endless. It is therefore conceivable that several distinct *in vivo* delivery carriers will be needed depending on the disease target and the route of administration. Among numerous delivery technologies currently being investigated for siRNA delivery, matrix systems can be regarded as a rather new strategy and their application in this field is still limited. Nevertheless, a number of matrices with diverging properties and dimensions have been proposed in an attempt to fulfill the needs for suitable siRNA delivery systems for a variety of applications. A recent approach to combine matrix with other delivery systems yielding hybrid systems with beneficial properties of both classes can also be observed. Although often not mentioned, preformulation of siRNA with cationic polymers and lipids yields polyplexes and lipoplexes respectively and basically results in a hybrid composite in which a known delivery system is incorporated in a matrix (Figure 2). In spite of using a biocompatible matrix system, the safety and biocompatibility of the used polymers and lipids is a concern towards clinical applications.

1.1 Macroscopic matrix systems

The use of macroscopic matrix systems is constricted to localized delivery due to their size. Several formulations are currently on the market based on topical application of macroscopic matrix systems for delivery of small, lipophilic molecules (e.g. controlled release of beta blockers from carbomer/polyvinylalcohol eye gels (Nyogel®)). Topical application of siRNA however, requires penetration enhancing techniques in order to reach therapeutic concentrations in the skin as the outermost layer of the epidermis, the stratum corneum, acts as a tight barrier for hydrophilic molecules larger than 500 Da [38]. When the barrier function of the skin is impaired, e.g. in the case of skin lesions, penetration of siRNA through the skin barrier is facilitated and topical delivery of siRNA is more effective. The use of physical or chemical methods to disrupt this barrier are consequently deemed necessary for siRNA delivery to the healthy skin and has already been exploited by Tran et al. under the form of nanoliposomes in combination with ultrasound for the treatment of cutaneous melanomas [39]. Thanik and colleagues used a hybrid system of a commercially available transfection reagent in combination with an FDA approved agarose matrix to efficiently silence mapk-1 and lamin A/C in a murine wound model [40].

Macroscopic matrix systems can also be applied for tissue engineering purposes. Tissue engineering deals with the reconstruction of degenerative tissues using three-dimensional cell-laden scaffolds, where morphogenesis is precisely induced and cell-matrix interaction is highly emphasized [41, 42]. Gradual release of naked or formulated (i.e. hybrid systems) siRNA from macroscopic matrices causing a continuous downregulation of undesired regulatory proteins or enzymes at the post-transcriptional level is indeed a promising application for siRNA. The potential of siRNA in regenerative medicine has already been illustrated by various applications for angiogenesis, wound healing and bone or nerve regeneration [41] but up to date, the use of matrix systems for these applications is still unexploited.

Matrix systems have also been proposed for the coating of stents in angioplasty. siRNA delivery from these stents could be used to reduce the proliferation and migration of vascular smooth muscle cells and intrastent restenosis through knockdown of key proteins such as matrix metalloproteinase 2 (MMP2). For this reason, San Juan et al. developed a cationized pullulan stent coating for arterial delivery of siRNA and applied the system *in vivo*. Although uptake of fluorescently labeled siRNA by the arterial tissue in proximity of the stents was shown, only a modest decrease of $28 \pm 13\%$ in MMP2 activity was observed in rabbits [43]. Such limited efficacy is to be expected as extracellular delivery of naked (i.e. unformulated) siRNA is considered a rather ineffective approach due to its inability to cross the cellular barrier without assistance [44-46]. Nevertheless, some clinical studies still utilize unformulated siRNA for local treatment of e.g. age-related macular degeneration. Although intraocular injection of free siRNA targeting vascular endothelial growth factor-A (VEGFA) or its receptor (VEGFR1) was shown to inhibit ocular neovascularization in mice [47, 48], angiogenesis suppression by siRNA was sequence and target independent and the result of toll-like receptor 3 (TLR3) activation on the surface of endothelial cells [49, 50]. Duxbury et al. showed however that a mammalian SID-1 homologue was able to enhance siRNA uptake and cause specific gene silencing in human cells [51] and a study by Wolfrum et al. indicated that this SID-1 homologue is at least in part responsible for the uptake of cholesterol conjugated siRNA in HepG2 cells [52]. In light of these results, there is a possibility that unformulated siRNA might be used in some cases through SID-1 mediated internalization of the siRNA in the target cells.

Krebs et al. prepared macroscopic collagen and alginate hydrogels for sustained delivery of siRNA/PEI and siRNA/chitosan polyplexes *in vitro* [53]. *In vivo* application of such macroscopic matrix systems is more

difficult as their introduction in the body via simple injection is not feasible due to their size. However, considerable progress has been made in the application of hydrogels that spontaneously gel under physiological conditions. These *in situ* gelling hydrogels can form at the site of injection avoiding the need for a more invasive introduction of the matrix system [54, 55]. To our knowledge, only Singh et al. exploited this approach by proposing an *in situ* crosslinking between dextran vinyl sulfone and tetra-functional polyethylene glycol thiol for delivery of both chemokines and DNA-siRNA loaded PEI-PLGA microparticles (approximately 1.5 μm in size). Phagocytosis of these microparticles by chemokine attracted immature dendritic cells could result in the delivery of immune-modulating siRNA and pDNA antigens to these dendritic cells [56].

1.2 Microscopic matrix systems

When compared to macroscopic systems, *in vivo* application of microscopic matrix systems for siRNA delivery is considered more feasible (i.e. through injection). Microscopic carriers with a size in the lower micron range are ideally suited for targeting cell types specialized in phagocytosis such as macrophages and dendritic cells (DC) [56-60]. Being the most potent antigen presenting cells, DCs have the unique ability to stimulate and polarize naïve T cells into either Th1 or Th2 phenotypes. In addition, they have a direct and indirect regulatory function of the immune response through induction of T regulatory cell generation and other tolerogenic mechanisms [61-64]. Therefore, RNAi in DCs could be a valuable strategy to modulate immune responses for e.g. treatment of auto-immune diseases [65]. Similarly, Brunner et al. proposed poly(D,L-lactide) (PLA) microspheres containing siRNA-PEI complexes for silencing of proinflammatory genes in peritoneal macrophages *in vivo* [58].

As their dimensions allow the potential incorporation of numerous siRNA molecules, microscopic delivery systems could be used as suitable depots for controlled release of siRNA. In this context, Raemdonck et al. proposed the use of biodegradable cationic dextran microgels for the controlled release of siRNA [66]. The kinetics of the siRNA release was shown to be dependent on the degradation rate of these microgels, which was in turn governed by the crosslink density of the gels. Although the biological functionality of this system was shown in a human hepatoma cell line, these carriers are probably more suited for siRNA delivery to strongly phagocytizing cell types.

PLGA microspheres for siRNA delivery with a size between 0.5 to 10 μm were prepared using a double emulsion evaporation method [67]. PLGA is an FDA approved biodegradable polymer and is used extensively in the field of drug delivery. The release profile of siRNA from these microspheres was characterized by an initial burst release of 10% and a subsequent release of approximately 5 percent during the following 250 hours (PBS, 37°C) [67]. This can likely be attributed to the slow degradation rate of PLGA, since PLGA microsphere erosion or degradation governs siRNA release. In addition, the low encapsulation efficiency of siRNA in PLGA is also considered a drawback and some concerns exist about the influence of the acidifying hydrolysis products (i.e. lactic and glycolic acid) of PLGA on the incorporated cargo [68, 69]. Recent studies have indicated however that the activity of siRNA remains high after formulating these molecules in PLGA matrices (Ref in press). Chemically modified PLGA has been synthesized in an attempt to circumvent some of the inherent shortcomings of PLGA based drug delivery [70]. A commonly used method to obtain a higher encapsulation efficiency of siRNA in PLGA matrices is by preformulating siRNA with a cationic polymer or lipid before encapsulation into hydrophobic PLGA. Murata et al. used arginine and PEI to increase the encapsulation efficiency of siRNA in PLGA microspheres and were able to show that intratumoral injection of this formulation inhibited tumor proliferation in mice [71]. Similarly, Imamura et al. used PLGA microspheres with PEI and siRNA against *Erc* to suppress tumor growth in a *Tsc2* mutant renal carcinoma model. However, the necessity of intratumoral injection for these formulations to work seriously limits their potential large scale application for the treatment of tumors.

1.3 Nanoscopic matrix systems

Nanosopic carriers have been receiving increasing attention over the last decade and they have some marked advantages when compared to microscopic carriers. For one, they can be easily introduced in the body via injection. Secondly, their smaller dimensions makes them ideally suited for the cellular delivery of therapeutics with an intracellular target and enables their uptake by cell types that are not specialized in phagocytosis, hereby rendering practically every cell type a possible target. Thirdly, their enhanced penetration of the extracellular matrix can also result in targeting tissues that are considered difficult to reach with larger carriers. Finally, their larger surface to volume ratio results in an improved and faster response to changes in their microenvironment which can be advantageous when developing stimuli responsive systems (10.1039/b915020j [72, 73]). In

contrast, this may also have a negative effect on the stability of these systems as decomplexation caused by e.g. interactions with blood components upon intravenous injection occurs more efficiently. However, compared to polyplex or vesicular systems, such nanoscopic matrices usually possess greater stability in complex media due to their crosslinked nature. Further stabilization of matrix systems can be achieved by surface modifications with hydrophilic molecules such as polyethylene glycol (PEGylation) and results in increased blood circulation times upon intravenous injection. An overview on the effects of PEGylation can be found in a review by Owens and Peppas [74].

Following the development of dextran microgels, our group prepared cationic dextran nanogels of about 200 nm in size using an inverse miniemulsion photopolymerization. The degradation kinetics of these nanogels can be tuned in a similar way as for the dextran microgels described above (3.2). It was demonstrated that the dextran nanogels could be loaded with high amounts of siRNA based on electrostatic interaction. Upon cellular internalization in human hepatoma cells, these nanogels were trafficked towards the endolysosomes. Endosomolytic techniques such as photochemical internalization (PCI) and the use of an influenza-derived fusogenic peptide (diINF-7) were shown to contribute significantly to luciferase gene silencing in human hepatoma cells [11, 75, 76]. Using a similar concept, these dextran nanogels were recently PEGylated by covalently attaching N-hydroxysuccinimidyl polyethylene glycol to increase their stability and potential as an *in vivo* applicable drug delivery system (in press).

Hyaluronic acid (HA) nanogels crosslinked with disulfide linkages were prepared by an inverse emulsion method by Lee and colleagues. SiRNA was physically entrapped within the 200 nm nanogels during the emulsion/crosslinking process and the degradation of the disulfide crosslinked HA nanogels and subsequent siRNA release could be triggered by the addition of glutathione, an intracellular reductive agent. The responsiveness of an siRNA delivery carrier to a reductive environment can be a valuable asset as these particles will selectively release their payload upon contact with the high concentrations of glutathione in the cell cytoplasm. Endosomal escape however remains an issue as the acidic non-reductive environment of the endosomes limits the cleavage of the disulfide linkages in the nanogels. The HA nanogels were shown to silence EGFP expression in HCT-116 cells up to 62 % in the presence of 10% fetal bovine serum. Furthermore, HA is known to regulate angiogenesis in many types of tumors and HA receptors such as CD44 and RHAMM are abundantly expressed on the surface of tumor cells. Using a competition experiment, the authors showed that

cellular uptake of these negatively charged matrix systems depended on the recognition of HA by CD44 receptors, indicating an inherent targeting of the nanogels to cells expressing CD44 [77].

As mentioned before, siRNA encapsulation efficiency in PLGA matrices is not very efficient due to the hydrophobic nature of PLGA. However, careful selection of the process parameters and the use of acetylated bovine serum albumin (BSA) to stabilize the water-oil interface, resulted in siRNA encapsulation efficiencies of up to 57% using a double emulsion solvent evaporation method [78]. Although it was recently shown that the siRNA released from PLGA nanoparticles was intact [78], transfection efficiencies of pure PLGA/siRNA nanoparticles are expected to be low. The high negative charge and slow degradation rate seriously limit the cellular uptake and intracellular siRNA release respectively, favoring the use of cationic moieties such as polyethyleneimine (PEI) to obtain a hybrid system with higher efficacy. However, as mentioned before these cationic moieties could result in a higher toxicity, in contrast with the FDA approved PLGA. The combination of PLGA and PEI has already been exploited for nanosized delivery systems by several authors. Patil and Panyam prepared PLGA-PEI containing nanoparticles using a double emulsion-solvent evaporation technique [79]. Addition of acetylated BSA improved siRNA encapsulation and release from these negatively charged nanoparticles [78]. Katas and colleagues used a modified emulsification diffusion method to generate PLGA-PEI nanoparticles for siRNA delivery with a size of around 100 nm [80].

In an excellent study by Woodrow et al., PLGA nanoparticles containing the naturally occurring polyamine spermidine and siRNA were used for intravaginal gene silencing in mice. The *in vivo* efficacy of these matrix systems was shown to be superior to a commercially available transfection agent. Fluorescent PLGA nanoparticles were shown to penetrate the vaginal tissue after vaginal instillation and were tolerated significantly better than the commercially available transfection agent. The fact that these matrix systems consist of FDA approved materials and the acidic hydrolysis products of PLGA are indigenous to the vaginal region, will likely be responsible for the fact that these nanoparticles are well tolerated. Knockdown of EGFP gene expression was sustained for at least 14 days using these simple matrix systems compared to 7 days using the commercially available transfection reagent.

Poly(N-isopropylmethacrylamide) (pNIPMAm) is an amphiphilic polymer strongly hydrated at physiological temperature. It undergoes an entropically driven coil-to-globule transition at approximately 43 °C and may be

useful in thermally triggered drug delivery. pNIPMAm was used for the development of core/shell nanogels with surface-localized peptides for active targeting purposes. Nanogel core particles were prepared using free-radical precipitation polymerization of N-isopropylmethacrylamide (NIPMAm) and N,N'-methylenebis(acrylamide) (BIS) and were subsequently used as seeds for the addition of a hydrogel shell composed of NIPMAm, BIS and aminopropylmethacrylate (APMA). The resulting core/shell non degradable nanogels were functionalized with YSA peptides that specifically target the Eph2A receptor and were loaded by resuspending the lyophilized nanogels in an aqueous siRNA solution. Preliminary investigations of gene silencing capacity illustrate an effective knockdown of epidermal growth factor receptors (EGFR) and an acceptable toxicity in an ovarian cancer cell line [81, 82].

Polyion complex (PIC) micelles are interesting candidates for drug delivery. These carriers are formed when a block copolymer with a neutral hydrophilic block and an ionic block is mixed with oppositely charged compounds. The resulting micelles have a core-shell structure with a core consisting of the polyion complexes and a shell consisting of the neutral block. The main driving force for the formation of these PIC micelles is the electrostatic attraction between the ionic block and counter-charged compounds [83]. Tamura et al. developed nanogels by chemically crosslinking the core of such PIC micelles and loading them with siRNA. These stabilized polyion complexes, about 110 nm in diameter, with a chemically cross-linked poly[2-(N,N-diethylaminoethyl)methacrylate] (PDEAMA) core surrounded by PEG tethered chains showed improved stability against polyanion exchange reactions when compared to non cross-linked PICs. This demonstrates the improved stability of matrix systems as mentioned earlier. SiRNA delivery was demonstrated by a firefly luciferase knockdown of 65% in human hepatoma cells after pre-incubating the nanogels in FBS [84].

Polyethyleneimine (PEI) polyplexes have already been used extensively for nucleic acid delivery . PEI mediated delivery is considered quite efficient due to improved endosomal escape of the polyplexes via the proton sponge effect [85]. One of the main disadvantages of PEI polyplexes are the rather high toxicity and low stability in complex media. Vinogradov et al. crosslinked poly(ethylene oxide) (PEO) and PEI to form undegradable nanosized PEO-*cl*-PEI nanogels that are less toxic and more stable than PEI polyplexes. When loaded with negatively charged oligonucleotides, screening of the positive charges causes the collapse of the gel network resulting in the efficient encapsulation of the cargo. Although initially designed for the delivery of antisense oligonucleotides, delivery of siRNA using PEO-*cl*-PEI nanogels is currently under investigation [86].

2. Conclusion and future outlook

In this review, a brief overview is given on the recent applications of matrix systems for siRNA delivery. Although matrix systems clearly have many advantages, the exploitation of matrix systems for siRNA delivery is still in its infancy when compared to lipoplexes or polyplexes. Their occurrence in different sizes and forms results in their application in a wide variety of fields. These versatile and often tunable carriers can have some specific advantages such as controlled siRNA release and improved stability when compared to polyplex or vesicular delivery systems and certainly deserve their place among suitable candidates for efficient siRNA delivery *in vivo*. However, further *in vivo* data are urgently needed to support such claims.

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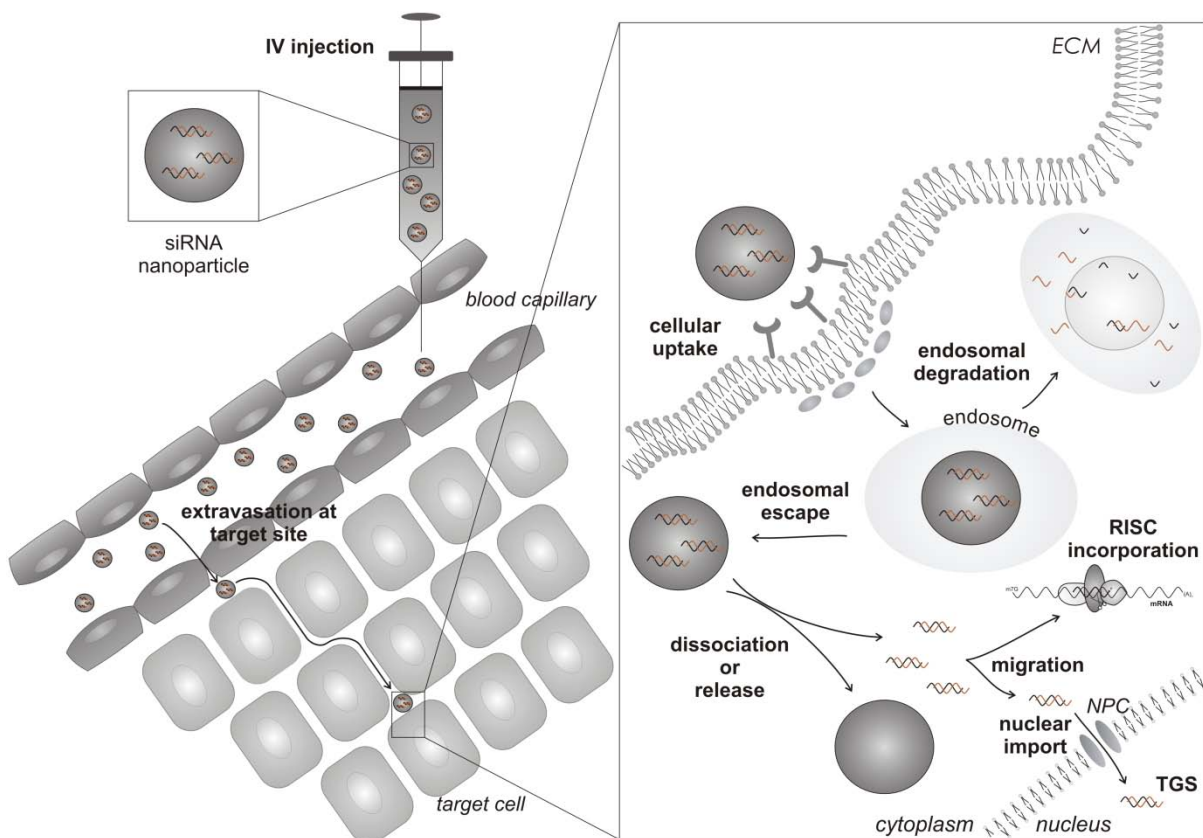
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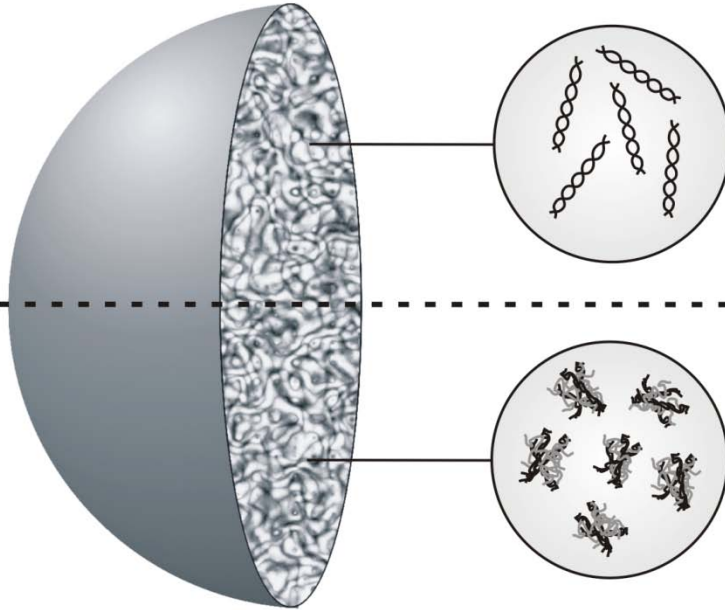
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Figure 1. Overview of biological barriers for siRNA therapy following intravenous (i.v.) injection. For in vivo application, siRNA can be formulated into nanosized carriers that should fulfil some basic requirements. They should be large enough to circumvent renal clearance, but small enough to be able to cross the capillary endothelium and accumulate in the target tissue. Moreover, the siRNA carrier has to be able to evade uptake by the mononuclear phagocyte system. Once the carrier has reached the target cell, it needs to deliver the siRNA to the cytoplasm. This usually involves cellular uptake through endocytosis, followed by escape from the endosome and carrier disassembly in the cytosol. ECM, extracellular matrix; NPC, nuclear pore complex; TGS, transcriptional gene silencing. Reprinted with permission from ref. 4. Copyright © 2008 Elsevier Ltd.

Figure 2. siRNA can be incorporated into matrix systems as such or alternatively precomplexed with lipids or polymers. The latter results in a hybrid system with beneficial properties of both matrices and lipoplexes or polyplexes.



Matrix + unformulated siRNA



Matrix + complexed siRNA = hybrid