Versatile electrostatically assembled polymeric siRNA nanovectors: Can they overcome the limits of siRNA tumor delivery?

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Versatile electrostatically assembled polymeric siRNA nanovectors: can they overcome the limits of siRNA tumor delivery?

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Keywords

siRNA delivery, electrostatically assembled polymeric siRNA nanovectors (EPSN), nanovector design, intracellular trafficking, protein down-regulation

Abbreviations

siRNA, small interfering RNA; EPSN, electrostatically assembled polymeric siRNA nanovectors; RNAi, RNA interference; shRNA, small hairpin RNA; miRNA, micro RNA; TLR, Toll-like receptor; N/P ratio, the molar ratio between the number of positive charges of polymer's amino groups and that of negative charges of siRNA's phosphate groups; SPION: superparamagnetic iron oxide nanoparticles; PEG, polyethylene glycol; PEI, Polyethylenimine; IL-4R, Interleukin-4 receptor; IL-4RPep-1, Interleukin-4 receptor binding peptide 1; CPP, cell-penetrating peptide; DMAEMA, [2-(dimethylamino) ethyl methacrylate]; BMA, butyl methacrylate; b-pDPB, b-(dimethylaminoethyl methacrylate-co-propylacrylic acid-co-butyl methacrylate); pD-b-pDPB, poly[dimethylaminoethyl methacrylate-b-(dimethylaminoethyl methacrylate-co-propylacrylic acid co-butyl methacrylate)]; CS-MSN, CPP-capped stealth magnetic siRNA nanovectorCPP

Abstract

The application of small interfering RNA (siRNA) cancer therapeutics is limited by several extra- and intracellular barriers including the presence of ribonucleases that degrade siRNA, the premature clearance, the impermeability of the cell membrane, or the difficulty to escape endo-lysosomal degradation. Therefore, several delivery systems have emerged to overcome these limitations and to successfully deliver siRNA to the tumor site. This review is focused on polymer-based siRNA nanovectors which exploit the negative charge of siRNA, representing a major challenge for siRNA delivery, to their advantage by loading siRNA via electrostatic assembly. These nanovectors are easy to prepare and to adapt for an optimal gene silencing efficiency. The ability of electrostatically assembled polymeric siRNA nanovectors (EPSN) to improve the half-life of siRNA, to favor the specificity of the delivery and the accumulation in tumor and to enhance the cellular uptake and endosomal escape for an efficient siRNA delivery will be discussed. Finally, the influence of the versatility of the structure of these nanovectors on the protein down-regulation will be evaluated.

Contents

1. Introduction

Since the 1990s and the discovery of post-transcriptional gene extinction in plants (Ratcliff et al., 1997), the mechanism of RNA interference (RNAi) has gained scientists' interest worldwide. This discovery has provided hope for the treatment of many severe diseases like cancers, autoimmune diseases, dominant genetic disorders and viral infections (Ferrari et al., 2012). RNAi is a natural phenomenon of sequence-specific gene silencing mediated by short sequences of non-coding endogenous RNA such as small hairpin RNA (shRNA), micro RNA (miRNA) and small interfering RNA (siRNA), considered as regulation systems of gene expression and RNA-based gene-silencing molecules (Carthew, Richard W. and Sontheimer et al., 2009). The use of RNAi is linked to the transfer of genetic material into damaged cells, in order to ensure a targeted molecular intervention and achieve a higher level of specific action than conventional cytotoxic chemotherapy (Jabir et al., 2012). Basically, thanks to the potency and the selectivity for the silencing of specific genes, RNAi-based therapy could treat any human disease caused by the over-expression of one or few genes (Aagaard and Rossi, 2007). Over the past few years, different approaches have been developed based on this strategy to inhibit the expression of certain genes, called oncogenes, coding for over-expressed proteins that are implicated in tumor growth (Haussecker, 2014). Among these small nucleic acids, it is generally accepted that siRNA sequences, of 21 to 23 nucleotides, offers the best combination of specificity and potency as a therapeutics and are the most used in the development of anticancer treatments (Ferrari et al., 2012; Resnier et al., 2013)

Following the demonstration of RNAi in mammalian cells in 2001 (Elbashir et al., 2001), several studies have quickly concentrated on specific gene silencing of siRNA to exploit this powerful mechanism to interfere with cancer-causing or cancer-promoting genes to develop a new class of drugs. In 2003, Song et *al.* presented the first *in vivo* evidence of RNAi-based therapeutic efficacy to protect mice from liver failure and fibrosis. By using siRNA duplexes targeting gene Fas, they demonstrated a specific decrease of mRNA level and protein expression of Fas in mice hepatocytes, after intravenous injections using a modified hydrodynamic transfection method (Song et al., 2003). Today, some siRNA-based therapies for cancer treatment are in clinical trial phases (Nikam and Gore, 2018; Tabernero et al., 2013; Tatiparti et al., 2017). For example, Silenseed Ltd performed the phase I clinical trial of its siRNA-based treatment (siG12D-LODER) against pancreatic cancer. SiG12D-LODER target the oncogene KRAS that is implicated in cancer growth. This trial was completed in 2014 and showed high safety and tolerability profiles of this treatment in patients. This therapy is currently in Phase II trial which aims to evaluate the efficacy of the combination of siG12D-LODER with standard chemotherapy treatment (Gemcitabine + nab-paclitaxel) by measuring progression-free survival in patients (Kaczmarek et al., 2017). The first siRNA-based therapy, ONPATTRO® (patisiran), was approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in August 2018 for the treatment of polyneuropathy of hereditary transthyretin-mediated amyloidosis in adult patients (Al Shaer et al., 2019; Rizk and Tüzmen, 2017). This approval presents a great achievement in nanomedicine discovery and development and provides hope for the progress toward an anti-cancer application.

Despite the therapeutic potency of siRNA, *in vitro* and *in vivo* trials revealed extra- and intracellular barriers difficult to overcome by naked siRNA. Therefore, different delivery systems have been exploited to increase the therapeutic potency of siRNA *in vivo*. The first part of this revue will present these barriers and the principle of siRNA nanovectorization. The second part of the review will focus on electrostatically assembled polymeric siRNA nanovectors (EPSN). First, the design of EPSN to overcome these barriers will be discussed, second, the parameters that have to be taken into account to evaluate the protein down-regulation efficiency will be presented and illustrated with actually studied EPSN.

2. siRNA delivery: challenges and nanovectorization

2.1. Extra- and intracellular barriers for naked siRNA

The limitations of naked siRNA are due to their properties (charge, hydrophilicity, size, sensitivity to degradation …) which represent hurdles in each step of the trafficking of siRNA, extra- and intracellularly (Figure 1). For example, in the blood or in biological environment, presence of enzymes such as ribonucleases affects the siRNA stability and involves their rapid degradation (Gavrilov and Saltzman, 2012). Even if siRNA escape enzymatic degradation in blood, their small size favors their rapid elimination by renal clearance. Therefore, the accumulation of siRNA in target site is a big challenge. Furthermore, the characteristics of the tumor tissue, such as i) the heterogeneous blood flow distribution and poor perfusion of inner region of solid tumor, ii) the dense intercellular matrix in this region, and iii) high hypoxia, acidity and interstitial fluid pressure, due to dysfunctional tumor lymphatics (Forster et al., 2017; Gillies et al., 1999; Heldin et al., 2004), restrict the uniform delivery of nanovectors to the tumors in sufficient quantities (Jain and Stylianopoulos, 2010). Once in the tumor site, siRNA must reach their target cells that express or overexpress the gene(s) of interest. Nevertheless, naked siRNA do not have the ability to distinguish target cells and they can act in the same way on normal cells and defective cells including unwanted off-target effects (Wang et al., 2017). Moreover, the negative charge of siRNA phosphate groups and their hydrophilicity limit their ability to cross cell membranes, because of the electrostatic repulsions between siRNA and the cell surface, negatively charged as well, and the impermeability of the lipid bilayer to hydrophilic molecules (Dominska and Dykxhoorn, 2010; Reischl and Zimmer, 2009; Videira et al., 2014). The small amount of siRNA that overcomes previously mentioned challenges and is internalized into cells must escape endosomal/lysosomal degradation in order to reach cytosol where its targets are present (Gavrilov and Saltzman, 2012). In addition to these limitations, the immunogenicity of siRNA represents another concern associated with *in vivo* administration. In fact, Reynolds *et al.* reported that siRNA can activate the innate immune reaction by inducing the expression of associated genes such as interferons or interferon-inducible genes. They demonstrated that this activation is cell type- and siRNA length-dependent (Reynolds et al., 2006). Other studies showed that certain siRNA can be recognized by some Toll-like receptors (TLR) such as TLR3, 7 and 8. This recognition can trigger interferon pathway responses (Behlke, 2006). The activation of this pathway results in the induction of the apoptosis and the cell death (from 20% to 60% of cell death) (Reynolds et al., 2006). The consequence of the association of previously presented limitations of the use of naked siRNA is an unsatisfactory effect *in vitro* as well as *in vivo*. It is, therefore, necessary to develop delivery systems with suitable properties to overcome all these challenges.

2.2. Nanovectorization for siRNA delivery

Because of the challenges mentioned above, many approaches were adopted to develop various galenic forms of siRNA-based medicine, in order to exploit the powerful effect of siRNA in anticancer therapies. One promising approach is the loading of siRNA in a nanovector. This strategy, called siRNA nanovectorization, consists in associating siRNA to suitable materials to obtain a nano-sized vector able to effectively convey siRNA toward their target (Ferrari et al., 2012; Resnier et al., 2013). Nanovectors are developed to carry and deliver drugs, oligonucleotides, peptides or other desired cargos to target tissues. Various nanosystems have been used for siRNA delivery in biomedical applications. At the present time, a relatively extensive arsenal of nanovectors has been proposed to administer siRNA without interfering with their silencing efficiency (Ozcan et al., 2015). In literature several types of nanovectors are described, including organic (lipid-based, polymer-based, peptide-based) (Resnier et al., 2013) and inorganic ones (based on the use of iron oxide, gold, quantum dots, …) (Conde et al., 2014). These nanovectors can be associated with siRNA using various methods: 1) conjugation, which needs chemical intervention and consists in covalently attaching siRNA to the nanovector components (Ding et al., 2014, 2012; Muratovska and Eccles, 2004), 2) encapsulation that is based on the loading of siRNA into a protective shell (liposomes or micelles, for instance) (Chen et al., 2012; David et al., 2012; Mokhtarieh et al., 2018) and 3) electrostatic bonds which aim to complex negatively charged siRNA with positively charged nanovector components (Bruniaux et al., 2017; Guruprasath et al., 2017; Liu et al., 2011).

3. Electrostatically assembled polymeric siRNA nanovectors (EPSN)

The common cause of the different challenges of siRNA delivery is their anionic character. This property generally considered as a disadvantage can be used to complex siRNA electrostatically and then at the same time create a nanosystem and hide the charge. Electrostatic complexation can be achieved with cationic polymers, peptides/proteins or cationic lipids. This review will more particularly relate to siRNA polymer-based nanovectors which are less described in the review literature than siRNA lipid-based nanovectors. We will distinguish four groups of EPSN: (A) EPSN containing only polymers and siRNA, (B) EPSN decorated with peptides, (C) EPSN containing an inorganic core, and (D) EPSN containing an inorganic core and decorated with peptides (Figure 2).

Electrostatic interaction has several advantages such as the ease and the rapidity of nanovector formulation. The use of electrostatic association avoids siRNA chemical modification and purification procedures that could affect their biological activity (Cavallaro et al., 2017; Conde et al., 2014). Electrostatic interactions need positively charged components in the nanovector to bind negatively charged siRNA. The stability of such an assembly depends on the number of charged groups on the molecules, therefore on the pH and the ionic strength of the environment. This strategy to load siRNA in nanovectors might be advantageous for the release of therapeutic agent. In fact, pH-sensitive components such as polymers or peptides are usually used for this assembly. This property can be useful at two levels: 1) in the tumor site and 2) in endosomes. As tumor tissues exhibit an acidic environment with a pH significantly lower than that of normal tissues, these components can allow a smart release. For example, the change of the pH induces a modification in the polymer charge density, leading to a pretty localized release of siRNA in the target site (Shakiba et al., 2017). In endosomes, the pH-variation is also exploited for facilitating the release of siRNA from nanovectors (Creusat et al., 2012, 2010; Nguyen and Szoka, 2012). This process is known as endosomal escape and will be more discussed later. Furthermore, the tumor environment is, often also characterized by hypoxia and the enrichment with free radical species. This difference can be exploited as well for siRNA delivery by using hypoxia-sensitive polymers (Perche et al., 2014). Many studies for siRNA nanovector development adopt these strategies of formulation.

Despite the ease and the speed of the preparation of electrostatically assembled nanovectors, their development needs serious work on the optimization of the formulation. Indeed, a critical point is the stability of these complexes in biological environment. As the electrostatic association between siRNA and cationic components is low, these complexes may disassemble too early if the formulation is not optimized (Creusat et al., 2010; Creusat and Zuber, 2008). Therefore, to successfully complex siRNA with polymers, there are some parameters to consider such as the components ratio and concentration (Richards Grayson et al., 2006). One can distinguish two types of ratio described in literature: i) charge ratio which represents the molar ratio between the number of positive charges of polymers (for instance, those of amino groups) and that of negative charges of siRNA phosphate groups (N/P ratio) (Guruprasath et al., 2017; Werfel et al., 2017), and ii) mass ratio which represents the ratio between the mass of polymer and that of siRNA (Corbet et al., 2016; Veiseh et al., 2010). These ratios are usually optimized at the beginning of each siRNA nanovector development to determine the best formulation. The complexation efficiency is often evaluated by gel retardation assay using ethidium bromide, as nucleic acid intercalant (Liu et al., 2011; Veiseh et al., 2011b, 2010). In fact, with this technique, free siRNA appear as fluorescent bands, while no fluorescence is detected if they are complexed and not accessible to ethidium bromide.

siRNA can also be complexed with polymers surrounding an inorganic core or support (Ben Djemaa et al., 2018; Guruprasath et al., 2017; Pittella et al., 2011). In this case another ratio appears which defines the quantity of the inorganic part used in the nanovector. The presence of the inorganic core can be advantageous for the formulation. In fact, it has been demonstrated that the use of the inorganic core (based on superparamagnetic iron oxide nanoparticles (SPION)) plays an important role in the stability and the control of the size of the final nanovector. Addition of an inorganic core to the formulation decreased the size and the polydispersity index of the complexes from 213nm to 175 nm and from 0.43 to 0.34, respectively (Ben Djemaa et al., 2018). Moreover, Xie *et al.* developed hybrid nanoparticles based on calcium phosphate core for the electrostatic loading and delivery of siRNA. These nanoparticles exhibited efficient siRNA loading and enhanced colloidal and serum stability (Xie et al., 2014). Apart from their role in the formulation and the transport of siRNA, some inorganic cores such as quantum dots (Derfus et al., 2007; Pan et al., 2010), gold nanoparticles (Jaganathan et al., 2014; Rosi et al., 2006; Song et al., 2010) or magnetic nanoparticles (Lu et al., 2007; Sun et al., 2008), can also be used as diagnostic tools. They allow the monitoring and the study of the distribution of inorganic nanovectors [45,46] using fluorescence, fluorescent energy transfer

(Fan et al., 2003) or magnetic resonance imaging (Pan et al., 2010; Sosnovik et al., 2008). Therefore, the choice of this inorganic part of the nanovectors is of great interest.

As an example of electrostatically assembled siRNA nanovectors, Pitella *et al.* presented a nanosystem based on a stable core of calcium phosphate nanoparticles coated with polyethylene glycol (PEG) and a charge-conversional polymer for the delivery of siRNA. This nanovector was prepared by simple mixing of the components at a determined concentration and was confirmed to possess excellent siRNA loading (about 80% of dose) (Pittella et al., 2011). Yet, Miteva and coworkers used two diblock polymers based on polyethylene glycol (PEG-b-pDPB) and polydimethylaminoethyl (pD-b-pDPB), for the nanovectorization of siRNA. Results showed a high cytoplasmic release and bioavailability in triple negative breast cancer cells (MDA-MB-231) due to the high intracellular unpackaging of the complex, quantified by FRET (Miteva et al., 2015). This electrostatic interaction shows a good balance between the siRNA complexation and release which present a suitable feature for siRNA delivery.

Certainly, this strategy of siRNA nanovectors formulation has many advantages but the electrostatic assembly results in less controlled structures in terms of components organization and nanovector size due to the poor control of their interactions and the formation of electrostatic bonds. One question rises here: are these siRNA nanovectors able to accomplish their mission to successfully transport siRNA through biological barriers and efficiently deliver them into tumor site to down-regulate the targeted gene(s) in cancer cells?

The main goal of siRNA nanovectors development is to improve the efficiency of used siRNA to down-regulate targeted genes. Therefore, to obtain a successful siRNA gene silencing, the nanovector must provide a) the protection of siRNA and the suitable stealthiness, b) the specific recognition of target cells or tissues, c) the capacity to cross cell membranes and d) the ability to escape endosomes and to deliver siRNA into the cytosol (Figure 3).

3.1. Design rationale of EPSN to overcome extracellular barriers

One of the principal needs of siRNA nanovectorization is to protect siRNA from biodegradation and to delay their elimination via clearance organs. Therefore, two properties can be brought to siRNA in order to improve the chance to reach their therapeutic target: physico-chemical stability and immune stealthiness.

3.1.1. Protection from enzymatic degradation and premature clearance

Actually, the presence of enzymes such as ribonucleases in biological environment threatens siRNA integrity and shortens their plasma half-life (Behlke, 2006). To solve this problem, one strategy is the electrostatic binding of siRNA with polymers. Table 1 presents some of the most used polymers in the development of siRNA delivery nanovectors. Polymers, especially biocompatible ones, have been considered as attractive materials for molecules delivery because of their interesting features (Gary et al., 2007; Tan et al., 2011; Venditti, 2017). In fact, polymers and polymer-based siRNA nanovectors show high colloidal stability in biological environment (Veiseh et al., 2011a) and have the ability to increase the half-life of siRNA in serum by limiting the accessibility of enzymes and molecules to siRNA (Arnold et al., 2017).

Cationic polymers are widely used for siRNA nanovector development strategies thanks to the presence of multiple positive charges per molecule and their ability to bind siRNA electrostatically (Liu et al., 2014; Veiseh et al., 2011b). In our previously published results, we showed that naked siRNA were degraded in the presence of a low percentage of serum (5%) after 4h and in the presence of ribonuclease A within 30 min. However, the use of two cationic polymers, chitosan and poly-L-arginine, in a siRNA nanovector offers a complete complexation and provides a protection of siRNA even in a high amount of serum (50%) or in the presence of ribonuclease A during 4 h (Ben Djemaa et al., 2018; Bruniaux et al., 2017).

Another example of cationic polymers classically used and studied for gene delivery/therapy is Polyethylenimine (PEI). PEI is a synthetic macromolecule consisting of a repeating amine and ethyl unit, with a high cationic charge density able to condense spontaneously, via electrostatic interaction, anionically charged siRNA and increase their stability in biological medium (Boussif et al., 1995). In their study, Liu et colleagues have successfully complexed siRNA with Alkyl-PEI. This complexation results in a high siRNA protection from enzymatic degradation in the presence of 50% of serum and at 37°C, evaluated by a qualitative gel retardation assay (Liu et al., 2011). In addition to protecting the siRNA from the degradation and the early elimination, these polymers are able to condense nucleic acid and increase the size of the complex, compared to the size of naked siRNA, to avoid the clearance of siRNA, while getting a relatively small size, suitable for gene delivery (Arnold et al., 2017; Parmar et al., 2018; Videira et al., 2014).

In most cases, the limitation of the use of cationic polymers with a high charge density, such as PEI, poly-arginine and poly-lysine, is the relative toxicity (Lv et al., 2006). Different studies reported that this toxicity depends on a set of factors such as the molecular weight, the dose and the degree of branching. In their study, Fischer and coworkers showed that the use of low molecular weight PEI (10 KDa) with a low degree of branching offers a good alternative for classic PEI and shows low cytotoxicity (Fischer et al., 1999). Ohsaki et al. reported that the use of poly-L-lysine with dendritic structure and several types of branch units did not show any significant toxicity in Hela cells (Ohsaki et al., 2002). One strategy used to reduce the toxicity is the chemical modification of these polymers such as lipid-substitution (Landry et al., 2012; Parmar et al., 2018), covalent conjugation (Foillard et al., 2011) or structural modification (Chiper et al., 2017; Fröhlich et al., 2012). Another strategy is the association of another polymer or copolymer like chitosan (Shim and Kwon, 2010), polyethylene glycol (Mi et al., 2005) or poly-(γ-benzyl l-glutamate) (Tian et al., 2007).

3.1.2. Increase of the immune stealthiness of siRNA

One of the major bottlenecks of the use of siRNA is their immunogenicity and their negative charge. These limitations underline the importance of an improved strategy for the delivery of siRNA. The complexation of siRNA with polymers could be an approach to overcome this challenge. Takeshita and coworkers used atelocollagen for the intravenous delivery of siRNA in a bone tumor metastasis model in mice. After the injection of a control naked siRNA or atelocollagen-siRNA complex, they evaluated the stimulation of the innate immune responses and they showed that the association of siRNA with this polymer did not result in an increase in the level of interferon (Takeshita et al., 2005). Moreover, the association of siRNA to polymers could neutralize their negative charge. As an example, the electrostatic assembly of siRNA with Alkyl-PEI or with a complex of polymers and peptide result in neutral zeta potential of the nanovectors around -2.6 or -0.01 mV, respectively (Liu et al., 2011; Veiseh et al., 2011b). However, the use of several cationic polymers or highly positively charged polymers results in unwanted high density of positive charges. In fact, a high positive surface charge induces the interaction with negatively charged plasmatic molecules and the formation of large aggregates that can be recognized by the innate immune system and promotes their elimination (Resnier et al., 2013). In EPSN containing an inorganic core, the association of neutral polymers such as PEG or polyvinylpyrrolidone (Pan et al., 2018) is often chosen to mask charges and to increase the stealthiness of siRNA nanovectors (Arnold et al., 2017). Neutral polymers are usually attached by covalent interaction to the inorganic core of the nanovector (Veiseh et al., 2011b), to cationic polymers (Xie et al., 2014) or to both of those (Veiseh et al., 2010). For example, in one study, the use of polylysine to develop a siRNA nanovector for the targeting of breast tumor-initiating cells yielded in positive zeta potential of 19 mV. In contrary, the addition of PEG to polylysine in another siRNA nanovector resulted in a surface charge of 0.5 mV. By masking the surface charge of nanovectors, PEG is able to avoid siRNA nanovectors' binding to plasma proteins, prolong their systemic circulation time, prevent their recognition by the immune system and promote an enhanced permeability and retention (EPR) effect in different types of tumors (Jabir et al., 2012; Owens III and Peppas, 2006). Sun *et al.* showed that PEGylation of their polymeric siRNA nanovector using PEG_{5k} or PEG_{6K} prolonged the circulation time in the blood 4-fold compared to free siRNA, by preventing protein adsorption on the surface (Sun et al., 2015).

3.1.3. Targeting of cancer cells

One additional major flaw of naked siRNA is their lack of specific recognition of target cells. Thus, it is unlikely that siRNA can be accumulated with a high concentration and for a sufficient period of time for deep penetration in the core of the tumor. To take advantage of enhanced permeability and retention (EPR) effect that allows the accumulation in the tumoral site by passive targeting (Resnier et al., 2013), siRNA can be associated to polymers to obtain complexes with adequate properties (50 nm < size < 250 nm and neutral charge). In this case, the obtained EPSN remain compatible with an intravenous administration (Arnold et al., 2017; Videira et al., 2014). In some cancers whose cells do not express any specific marker or receptor, this passive targeting is the only hope for tumor accumulation of the nanovectors.

When active targeting is possible, one can improve the cellular specificity of siRNA nanovectors and increase their accumulation in the tumor. Therefore, biological ligands such as antibodies (anti-HER2 (Goren et al., 1996), anti-CD19 (Menezes et al., 1998)), peptides (Schmohl et al., 2017), vitamins (folate) (Dohmen et al., 2012), growth factors, enzymes are associated to nanovectors (Prokop and Davidson, 2008). Mostly, ligands are chosen for their high ability to target selectively some specific extracellular molecules (such as receptors) over-expressed in some tumor types (An et al., 2015; Lee et al., 2016a, 2016b). This review focuses on the use of peptides for the functionalization of EPSN, as these ligands can be useful at various levels during the extra- and/ or intracellular trafficking of the siRNA nanovector. Peptides are able to allow the active targeting of tumors as described above and/ or to participate in the cellular trafficking which will be discussed in the following sections of this review.

Peptides, or polyamines, are short chains containing less than 50 amino acids monomers linked by amide bonds and are structurally similar to proteins. Peptides can be found naturally or synthetically and have the potential for the stabilization and biofunctionalization of nanoparticles (Conde et al., 2014; Zhang et al., 2016). Peptides can be associated with siRNA nanovectors by electrostatic or covalent bonds (Corbet et al., 2016; Jiang et al., 2012; Muratovska and Eccles, 2004; Wang et al., 2009). Table 2 presents some of the most used peptides in the functionalization of siRNA nanovectors. Some peptides can be selectively addressed to membrane molecules on the surface of specific cells (Conde et al., 2014; Schmohl et al., 2017). Thus, the use of these peptides in siRNA nanovectors could guide and improve the interactions with cell surfaces. In order to treat cancer, Guruprasath and colleagues presented an example of the functionalization of siRNA nanovectors with peptide for active targeting. In this study, they demonstrated a specific interaction of their siRNA nanovector functionalized with Interleukin-4 receptor (IL-4R)-binding peptide 1 (IL4RPep-1) with the IL-4R up-regulated on cancer cells. Furthermore, they showed an efficient accumulation in tumor, 3-fold more than with nanovectors without peptide (Guruprasath et al., 2017).

To enhance the targeting and the penetration in the tumor site, a rational design of nanovectors that considers tumors characteristics and the properties of their microenvironment, mentioned in section 2.1., is needed. As solid tumors exhibit low interstitial pH, many pH-sensitive nanovectors were developed to deliver siRNA to tumors using pH-sensitive peptides for instance (Mok et al., 2010; Zhu et al., 2015). In addition, the formulation of hypoxia-sensitive nanovectors using, for example, hypoxia-responsive polymers or hypoxia-targeted polymers can be used to benefit from the hypoxia in tumor site (Kang et al., 2016; Perche et al., 2016). Perche and colleagues synthesized hypoxia-sensitive polymers to develop a nanovector for the delivery of siRNA in tumors. They showed that these polymers respond to the hypoxia-stimulation by detaching PEG from the complexes to enhance the accessibility and the targeting of tumor cells (Perche et al., 2014). In tumors, the deep penetration of nanovectors can also be achieved by the application of an external magnetic field thanks to the presence of iron magnetic nanoparticles in the formulation of the nanovector (Scherer et al., 2002).

3.2. Design rationale of EPSN to overcome intracellular barriers

3.2.1. Cellular uptake of EPSN

As the plasma membrane is negatively charged, it is important to load siRNA in positively charged or neutral nanosystems. Therefore, EPSN can be a good candidate for the nanovectorization of siRNA and an asset for the intracellular delivery. Thanks to the positive charge density of cationic polymers, they can easily favor the interaction with the cell membrane and facilitate the passage into the intracellular compartment (Cavallaro et al., 2017)

3.2.1.1. Internalization by endocytosis

As siRNA nanovectors are bigger than 1 kDa, cells use a variety of specialized internalization mechanisms to adapt their entry (Bareford and Swaan, 2007). Various internalization mechanisms can be observed depending on nanovector characteristics and the nature of its components. Endocytosis is the principal pathway implicated in the entry of nanoparticles into cells. This process involves the transport of extracellular molecules/particles into cells by vesicles derived from the invagination of the plasma membrane. Generally, endocytosis occurs by different mechanisms which can be categorized in two groups: phagocytosis (to clear large pathogens or large cell debris) characterize only mammalian specialized cells like macrophage, while pinocytosis (the uptake of fluid and solutes) takes place in all cells. There are four pinocytosis mechanisms differing with regard to the size of the endocytic vesicle, the nature of the molecule and the mechanism of vesicle formation: 1) clathrin-mediated endocytosis (vesicles ~120 nm), 2) caveolae-mediated endocytosis (vesicles ~60 nm), 3) clathrin- and caveolae-independent endocytosis (vesicles ~90 nm) and 4) macropinocytosis (vesicles >1 µm) (Conner and Schmid, 2003; Marsh and McMahon, 1999) (Figure 4).

For EPSN constituted of siRNA complexed to polymers with or without inorganic core (Figure 2, A and C), passive endocytosis is expected. Werfel *et al.* showed that cells treated with siRNA nanovector prepared with a combination of [2-(dimethylamino) ethyl methacrylate] (DMAEMA) copolymerized with butyl methacrylate (BMA) and pre-conjugation of PEG and DMAEMA (DB-PD ternary si-NPs), with a zeta potential of 18 mV, exhibited a high fluorescence intensity of nanoparticles. This result showed the ability of this cationic block of polymer to enhance the cell internalization of the siRNA nanovector (Werfel et al., 2017). Similarly, Cavalieri and colleagues designed a siRNA nanovector prepared with poly-L-lysin and PEG for the silencing of the anti-apoptotic gene, survivin, in prostate cancer cells. In this study, they showed a rapid cell uptake of the siRNA nanovector occurred within 2 h in almost 100 % of cells. Moreover, they observed, using deconvolution fluorescence microscopy, that the siRNA nanovector was internalized by endocytosis (Cavalieri et al., 2015).

The functionalization of the surface of EPSN with peptides can help to enhance passage through the membrane mediated by active endocytosis (Azevedo et al., 2018) (Figure 2 B and D). Some peptides used for the functionalization of siRNA nanovectors are able to recognize specific molecules on the cell membrane such as receptors. Upon binding to these molecules, the entry of associated siRNA nanovector occurs by receptor-mediated endocytosis. This internalization pathway is largely used for active targeted siRNA delivery. In this process, receptors are considered as mediators between cells and extracellular molecules/particles, they play a crucial role in cellular internalization by ensuring high specific interaction. Although numerous mechanisms of ligand-receptor internalization exist, all occur by ligand-stimulated manner. Briefly, the binding of ligand, held on nanovectors surface, to the extracellular domain elicits the receptor phosphorylation. Following this step, the phosphorylated receptor-ligand binary complex or only the phosphorylated receptor is internalized (Allen, 2002). In the case of nanovectors, it is requested to be receptor-ligand internalization. Depending on ligand nature and cell type, intracellular processing of ligand can differ. Although internalized ligands (likewise peptide functionalized nanovectors) commonly end into endosomal compartment, receptor is recycled back to the cell membrane (Lodish et al., 2000; Prokop and Davidson, 2008). Indeed, in endosomes, the recruitment of vacuolar ATPase pump causes vesicles acidification by the entry of H^+ ions. The acidic pH induces a conformational change of receptors, often resulting in a ligand-receptor dissociation (Bareford and Swaan, 2007). This mechanism can be considered as the best entry route for a high targeting specificity and an efficient cellular uptake of nanovectors. As an example for this entry pathway, Guruprasath and coworkers functionalized their siRNA nanovector by IL-4 receptor-binding peptide (IL4RPep-1) to target IL-4R for the delivery of anti-Bcl-xL siRNA. Results showed a high accumulation of the siRNA nanovector in the tumor and a specific internalization by IL-4 receptor-mediated endocytosis (Guruprasath et al., 2017).

3.2.1.2. Internalization mediated by transcytosis

EPSN can also be decorated with some peptides to enhance the internalization thanks to their ability to cross the cell membrane by a non-endocytic pathway, transcytosis (Figure 2 B and D). It is particularly interesting when active targeting is not possible, like when the cells do not over-express any specific receptor.

Transcytosis is a mechanism allowing to cross the cell membrane in an energy independent way. It depends on the size, the charge and the nature of nanovector surface components and on the nanovector concentration (Tuma and Hubbard, 2003). Peptide-functionalized nanovectors, in particular those conjugated to cell-penetrating peptides (CPP), have various internalization mechanisms. CPP are short peptide sequences of about thirty amino acids positively charged and are known for their ability to cross the lipid membrane by translocation mediated with their hydrophobic sequence and directly enter the cytosol (Rothbard et al., 2004). Briefly, the amphipathic character and the easy change of CPP structure from α-helices to β-sheets provide this peptide a high degree of conformational flexibility. This property has a key role in CPP translocation capacity. CPP – mediated transcytosis is induced by CPP hydrophobic extremity, so-called membrane perturbing/interacting domain. This extremity initiates lipid destabilization of cell membranes which permits the fusion with lipid bilayer in order to gain the cytoplasmic compartment (Galdiero et al., 2015). These short amphipathic peptides are emerging as attractive gene delivery tools and they can be associated with other molecules of different nature such as polymers (Wang et al., 2014). One example of the application of such a short peptide was published by Oh *et al*. who used the CPP R3V6 associated by electrostatic manner to deliver siRNA against sphingosine-1-phosphate lyase (S1PLyase) and recombinant high mobility group box-1 box A peptide (HMGB1A) into LA-4 lung epithelial cells in animal model. The presence of R3V6 increases the cell entry of the nanovector (Oh and Lee, 2014). Despite the absence of specific tumor recognition, this study showed that the use of CPP improves siRNA delivery, indicating the participation of the EPR effect. Once nanovectors are accumulated, the CPP intervenes to enhance the deep penetration into tumor cells. Veiseh and coworkers have evaluated PEG-modified iron oxide nanoparticles coated with an oligo-arginine and loaded with siRNA (size about 50 nm) for their cellular entry pathway in three types of cancer cells. Results showed an enhanced internalization of this siRNA nanovectors by transcytosis without the formation of endocytic vesicles (Veiseh et al., 2011b).

3.2.2. Endosomal escape

Due to their endosomal buffering ability, cationic polymers can facilitate the endosomal escape of siRNA. Most EPSN are internalized by endocytosis, more precisely pinocytosis (Corbet et al., 2016; Xie et al., 2014; Yin et al., 2016). Briefly, after immobilization on the cell surface, nanovectors are encompassed in vesicles derived from local invagination of the cell membrane. After vesicles formation, nanovectors are attracted into cell inside newly formed endosomes (Wang et al., 2010). In this stage, the challenge of siRNA nanovectors is to escape endosomes before their fusion with lysosomes to avoid degradation and to pass into the cytosol. At this level, cationic polymers could be good candidates for this challenge. In 1997, Behr and others introduced the concept of the proton sponge and hypothesized that polymers such as PEI, polylysine and polyarginine could buffer the acidity of endosomes and induce their rupture (Behr, 1997). Afterward, this concept was more studied and developed. To summarize, endosomes acidification causes two complementary and simultaneous effects. The first is the so-called “proton sponge effect” which consists of a massive entry of water following a high concentration of hydrogen chloride (HCl) caused by the stimulation of the flow of chloride ions after the increase of the H^+ ions density in endosomes. The second is the consequence of the acidification of the endosomes and is called the umbrella effect that occurs by the capture of positive charges by cationic components of nanovectors, inducing thus an increase in the volume occupied by these molecules caused by the repulsions between groups of the same charge. These two phenomena combined allow the lysis of the endosomes (Nguyen and Szoka, 2012) and promote the passage of nanovectors and/ or siRNA into the cytosol. Recently, the proton sponge hypothesis was discussed on the part of the lysis of the endosomal membrane. Several studies showed that this complete rupture is highly unlikely and that in the presence of cationic polymers, the endosomal escape is promoted by the interaction of polymers’ amino groups and the inner side of the membrane. This interaction causes a local membrane destabilization which leads a transient formation of “nanoscale holes” which could explain the endosomal escape (Jonker et al., 2017; Rehman et al., 2013; Schubert et al., 2018; Trützschler et al., 2018). In their study, Xie *et al*. used an inorganic core of calcium phosphate and a polymer coating (PEG and modified chitosan) to nanovectorize siRNA. They demonstrated that the nanovector was internalized mainly by macropinocytosis with the contribution of clathrin- and caveolae-mediated endocytosis. Using fluorescently labeled siRNA loaded in their nanovector, endosomal-lysosomal tracker and confocal laser scanning microscopy, they observed colocalization between the fluorophore associated to siRNA and that of the tracker after 3 h of nanovector incubation with cells. However, after 6 h the colocalization of these fluorescent signals was decreased, and fluorescent siRNA was detected in the cytoplasm. Authors explained this observation by the dissociation of the calcium phosphate core from polymers due to the protonation of amino groups of PEG-chitosan in the acidic environment. This process leads to the swelling of endosomes and then the release of siRNA into the cytoplasm

(Xie et al., 2014). Table 3 shows the entry pathway of nanovectors and the studies performed to investigate the endosomal escape by indicating the used techniques and the main results.

3.3. Evaluation of protein down-regulation efficiency

The efficiency of siRNA nanovectors is evaluated by the cellular and/or the molecular responses of treated cells or tissues and it depends on the used siRNA. The evaluation of the molecular response can reflect the efficiency of the nanovector even if there is no cellular effect of the used siRNA. Molecular responses are the inhibition of the targeted mRNA expression and consequently a decrease in the expression of the associated protein. In the development phase of a nanovector, model siRNA (or reporter siRNA) targeting GFP or luciferase are widely used because they are convenient, relatively inexpensive, and gives quantitative and rapid measurements. These siRNA are commonly used as a tool to study gene expression at the transcriptional level and they give a molecular response due to the inhibition of the GFP or the luciferase protein and the extinction of their signals, easily detected by flow cytometry (for GFP) or luminescence (for luciferase) analysis. In the validation phase, the cellular response is usually an induction of cell death and it is detected by cytotoxicity (WST-1, MTT, LDH, …) or apoptosis assays (Annexin V- FITC / PI assay, DNA laddering, …). The used siRNA usually target mRNA of genes implicated in different functions needed for tumor process such as cell survival (survivin (Cavalieri et al., 2015)), apoptosis control (Bcl-2 family (Guruprasath et al., 2017)), cell cycle control, tumoral growth and angiogenesis (HIF 1α (Zhu et al., 2015)), tumor cells migration, metastasis (VEGF (Chen et al., 2014)), etc. The efficiency of a nanovector depend on a) the nanovector design and composition, b) the chosen cellular model and the corresponding target protein and c) the chosen therapeutic scheme. Tables 5 and 6 give an overview of some examples of existing versatile polymeric nanovectors which efficiently down-regulate protein expression.

3.3.1. Influence of the nanovector design and composition

To obtain a high gene silencing, it is necessary to carefully design the siRNA nanovector considering all the challenges presented above. Veiseh and colleagues developed a nanovector for nucleic acid delivery based on the use of a magnetic nanoplatform of SPION core coated with a copolymer of chitosan-grafted-PEG and PEI. In this nanosystem, the use of the combination of chitosan and PEG stabilized the nanovector. Cationic PEI was incorporated into this coating to protect and complex, by electrostatic interaction, negatively charged oligonucleotide (Veiseh et al., 2009). In a following study, they improved the specific targeting of the nanovector using a biological ligand, the chlorotoxin peptide. The addition of this peptide enhanced the cell internalization of the siRNA nanovector by receptor-mediated endocytosis pathway and its ability to escape endosomes (Veiseh et al., 2010). This nanovector exhibited a high accumulation in the tumor, after systemic administration, and showed an increased transfection efficiency in a mouse model of glioma compared to nanovectors without chlorotoxin peptide (Kievit et al., 2010). This nanovector is a good example of siRNA nanovector in which components were well chosen and each one has a key role and a specific function.

Several studies showed that the chosen polymers could affect the stability, the trafficking and, therefore, the efficiency of the siRNA nanovector. For example, siRNA nanovectors containing PEG as a neutral polymer to increase their colloidal stability and their stealthiness show, generally, a transfection efficiency higher than 60% (Cavalieri et al., 2015; Miteva et al., 2015; Werfel et al., 2017; Xie et al., 2014). Veiseh and coworkers evaluated PEG-modified iron oxide nanoparticles coated with either polyarginine, polylysine or PEI for their ability in promoting gene knockdown by siRNA delivery. They demonstrated that the transfection efficiency depended on the used cationic polymer. In fact, it was inferior to 40% by using polylysine or PEI as the only cationic polymer in the formulation. However, the replacement of these two polymers by polyarginine increases the efficiency of the nanovector to 68% (Veiseh et al., 2011b). In other studies, the use of PEI and polylysine in siRNA nanovectors with more complex structures showed a high down-regulation efficiency. For example, the use of PEI with chitosan, PEG and a small peptide (Ragelle et al., 2015) or with SPIONs, chitosan, PEG and chlorotoxin peptide (Veiseh et al., 2010) result in, respectively, 80% and 62% of GFP down-regulation. Similarly, the use of polylysine with modified PEG (Cavalieri et al., 2015) or with PEG, polyarginine and quantum dots (Zhu et al., 2015) results in 60% of transfection efficiency.

3.3.2. Influence of the cellular model and the corresponding target protein

Model cells used to evaluate the down-regulation efficiency of siRNA nanovectors are always chosen to be representative of the targeted cancer type (Table 4). The cellular responses towards gene therapies depend on the cell type. In this context, Veiseh *et al.* evaluated *in vitro* the transfection efficiency of EPSN based on the use of PEGylated superparamagnetic iron oxide nanoparticles (SPION) polyarginine in cell lines expressing GFP representative of glioma, breast cancer and colon adenocarcinoma: C6, MCF7, and TC2 respectively. These nanovectors appear to be significantly more efficient to down-regulate the expression of the GFP in MCF7 cells (68.2 %), followed by C6 cells (52.9%) and TC2 cells (24%) (Veiseh et al., 2011b). Similarly, Werfel and coworkers showed that the transfection efficiency of a siRNA nanovector formulated using DMAEMA, BMA and PEG as polymers and siRNA anti luciferase at a concentration of 100 nM varies in three cell lines: MDA-MB-231, NIH3T3 and mesenchymal stem cell (MSC), but it was higher than 80 % in all the cell lines (Werfel et al., 2017).

As we mentioned above, generally, in the development stage of siRNA nanovectors it is easier to use a model gene, but then it is necessary to evaluate the silencing potential of the siRNA nanovector on a target gene, usually related to the tumor process (Table 4 and Table 5). However, the modification of the target protein leads, sometimes, to a variable down-regulation efficiency dependent on the protein. For example, Xie *et al.* evaluated the transfection efficiency of a siRNA nanovector prepared with an inorganic core of calcium phosphate nanoparticles and a coating of PEG grafted carboxymethyl chitosan on HepG2 model cells expressing luciferase at a siRNA concentration of 100 nM. The incubation of this nanovector prepared with siRNA anti-luciferase leads to 79% of silencing efficacy. However, the evaluation of the therapeutic potential of siRNA delivery targeting hTERT gene results in only 60% and almost 50 % of inhibition in the targeted mRNA and protein level (Xie et al., 2014). This study showed a loss of down-regulation efficiency of at least 20% between the model gene and the gene of interest.

3.3.3. Influence of the therapeutic scheme

3.3.3.1. Dose of siRNA

The dose or the concentration of siRNA is one of the important parameters to consider for successful gene transfection and satisfactory gene silencing (Table 4 and Table 5). The determination of the adequate siRNA quantity requires an optimization step. Ragelle and colleagues performed a transfection of cells with an EPSN at different siRNA concentrations (from 12.5 nM to 200 nM). They showed that at low concentration (12.5 – 50 nM) the gene silencing of GFP was lower than 40% and it increased significantly up to 150 nM of siRNA to achieve almost 90%. However, no significant increase in the silencing efficiency was observed at concentrations above 150nM (Ragelle et al., 2015). Moreover, the used concentration of siRNA depends on the used nanovector. In fact, by using different EPSN the same down-regulation efficiency can be achieved, but with different siRNA concentrations. For example, to obtain 80% of silencing of luciferase in breast cancer cells, Liu and colleagues used 6 pmol of siRNA loaded in a nanovector based on iron oxide nanoparticles and alkyl-PEI (Liu et al., 2011). However, for the same luciferase silencing efficiency (80%), Miteva *et al.* used a siRNA nanovector prepared with two polymer blocks (PEG-b-pDPB et pD-b-pDPB) at a siRNA concentration of 100 nM (Miteva et al., 2015), much higher than the previous study. Likewise, the intravenous administration of vectorized siRNA (with DMAEMA, BMA and PEG) in a xenograft mouse cancer model at a concentration of 1 mg/kg resulted in 59 % of efficiency (Werfel et al., 2017). Yet, Corbet *et al.* obtained almost the same efficiency (60%) by injecting by the same route a siRNA nanovector prepared with two polymers, PEG and chitosan, and functionalized with the peptide RGD in a xenograft mouse cancer model at a dose twice as high (2 mg/kg) (Corbet et al., 2016). Therefore, the dose of siRNA must be adapted to the used system. That means that it is not the use of more siRNA that increases the silencing efficiency of the nanovector as shown in these two following studies. Ragelle *et al.* showed a knockdown of targeted gene expression (GFP) of 80% using their siRNA nanovector composed of three polymers: PEG, chitosan and PEI, and functionalized with RGD peptide in GFP model cells, at a siRNA concentration of 100 nM (Ragelle et al., 2015). However, Veiseh *et al.* used more than twice as much siRNA in a nanovector based on SPION, PEGylated chitosan and PEI and functionalized with a tumor-targeting peptide to obtain a GFP silencing efficiency of 62% (Veiseh et al., 2010).

3.3.3.2. Treatment time and administration protocol

For an efficient siRNA transfection *in vitro*, it is important to consider a sufficient treatment time, long enough for the internalization of the siRNA nanovector (Table 4). As an example, Cavalieri and colleagues exposed PC-3 cells to a nanovector prepared with anti-survivin siRNA for 72 h. After this treatment time, they obtained a negligible down-regulation of the protein survivin (<10%). The increase of the incubation time of siRNA nanovector with cells from 72 h to 120 h resulted in a marked silencing in the targeted gene (almost 60 %) (Cavalieri et al., 2015). Similarly, in a previous study published by our team, it was shown that the optimization of the treatment time of MDA-MB-231 cells expressing GFP with CS-MSN could improve the inhibition efficiency of the expression of GFP. An increase of the silencing of the targeted protein up to 4 h of treatment and the prolongation of this time did not improve the efficiency (Ben Djemaa et al., 2018). For *in vivo* studies, the treatment time can be translated by the administration protocol (i.e. number of injections and interval between injections). Many administration protocols with different numbers of injections and different administration schemes were described in the literature (Table 5). Tingjie *et al.* injected a siRNA nanovector 17 times (every other day for 34 days) (Yin et al., 2016). However, Werfel and colleagues administered their siRNA nanovector twice with an interval of 24 h (Werfel et al., 2017). In both studies, they obtained almost 60% of efficiency.

3.3.3.3. Routes of administration

The choice of the administration route depends on the accessibility of the tumors. In fact, for the tumors with deep localization such as liver cancer (Xie et al., 2014; Zhu et al., 2015), the only way to get access to them is through intravenous administration. However, it is possible to use both systemic or local administration (intravenous or intra-/peri-tumoral (Liu et al., 2011)) for the easy to access tumors such as breast cancer. For the intravenous administration, siRNA nanovectors have to overcome all biological barriers described in section 2.1. However, by using the intratumoral injection the nanovector is directly administrated into the tumor and only the cellular barriers needed to be overcome. Various routes of administration depending on the cancer type have been used (Table 5). In research from Xie and coworkers, the intravenous injection of siRNA at 1.2 mg/kg loaded in a nanovector composed of polymers and calcium phosphate core, in a xenograft liver cancer model showed an inhibition of approximative 60 % in tumor growth (Xie et al., 2014). As an example of local treatment, the intratumoral administration of vectorized siRNA at 250 pmol was applied by Liu *et al.* in xenograft breast cancer model for *in vivo* evaluation of the down-regulation efficiency of luciferase. Results showed a significant reduction of the luciferase expression in the tumor (Liu et al., 2011). Yet, for the treatment of xenograft carcinoma mouse model, Corbet and colleagues used both intravenous and peritumoral route to deliver a combination of vectorized therapeutic siRNA. This treatment led to a dramatic tumor growth inhibition (about 60%) upon peritumoral but also systemic administration.

4. Summary and concluding remarks

In summary, an interesting approach to overcome the extra- and intracellular barriers for the delivery of naked siRNA is the use of electrostatically assembled polymer-based nanovectors. One advantage of EPSN is their versatility due to their easy and rapid preparation. Nevertheless, the development of EPSN require a careful optimization (amount of the different components, siRNA complexation; physico-chemical characteristics). To obtain a high efficacy, each component has to be well-chosen and plays a specific role to overcome these barriers: (i) polymers complex and protect siRNA from enzymatic degradation and premature clearance, (ii) neutral polymers increase the immune stealthiness and the circulation time in blood (iii) cationic polymers are implicated in the cellular internalization and in the endosomal escape, (iv) targeting peptides and cell-penetrating peptides enhance the tumor targeting and the uptake respectively, and (v) an inorganic core can be used for diagnostic purpose and to improve the physico-chemical characteristics. In addition, adequate properties of EPSN can enhance the accumulation in the tumor site due to the EPR effect.

Furthermore, the siRNA sequences need to be carefully chosen for an efficient silencing and to avoid the off-target effect of siRNA. Besides the formulation of EPSN, the silencing efficiency of EPSN depends on other factors related to the application of the treatment such as cell line, targeted protein, siRNA dose, treatment time, administration route, etc.

In conclusion, EPSN have proved their ability to successfully deliver siRNA into tumor cells and appear as a promising tool for cancer treatment. However, there is still much progress needed to reach clinical trials and achieve this goal.

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Conflicts of interest

Authors declare that there are no conflicts of interest in the present manuscript.

References

Aagaard, L., Rossi, J.J., 2007. RNAi therapeutics: principles, prospects and challenges. Adv. Drug Deliv. Rev. 59, 75–86. https://doi.org/10.1016/j.addr.2007.03.005

Al Shaer, D., Al Musaimi, O., Albericio, F., de la Torre, B., Al Shaer, D., Al Musaimi, O., Albericio, F., de la Torre, B.G., 2019. 2018 FDA Tides Harvest. Pharmaceuticals 12, 52. https://doi.org/10.3390/ph12020052

Allen, T.M., 2002. Ligand-targeted therapeutics in anticancer therapy. Nat. Rev. Cancer 2, 750–763. https://doi.org/10.1038/nrc903

An, S., He, D., Wagner, E., Jiang, C., 2015. Peptide-like Polymers Exerting Effective Glioma-Targeted siRNA Delivery and Release for Therapeutic Application. Small 11, 5142–5150. https://doi.org/10.1002/smll.201501167

Arnold, A.E., Czupiel, P., Shoichet, M., 2017. Engineered polymeric nanoparticles to guide the cellular internalization and trafficking of small interfering ribonucleic acids. J. Control. Release 259, 3–15. https://doi.org/10.1016/j.jconrel.2017.02.019

Azevedo, C., Macedo, M.H., Sarmento, B., 2018. Strategies for the enhanced intracellular delivery of nanomaterials. Drug Discov. Today 23, 944–959. https://doi.org/10.1016/J.DRUDIS.2017.08.011

Bareford, L.M., Swaan, P.W., 2007. ENDOCYTIC MECHANISMS FOR TARGETED DRUG DELIVERY. Adv. Drug Deliv. Rev. 59, 748–758. https://doi.org/10.1016/j.addr.2007.06.008

Behlke, M.A., 2006. Progress towards in Vivo Use of siRNAs. Mol. Ther. 13, 644–670. https://doi.org/10.1016/J.YMTHE.2006.01.001

Behr, J.-P., 1997. The Proton Sponge: a Trick to Enter Cells the Viruses Did Not Exploit. Chim. Int. J. Chem. 51, 34–36.

Ben Djemaa, S., David, S., Hervé-Aubert, K., Falanga, A., Galdiero, S., Allard-Vannier, E., Chourpa, I., Munnier, E., 2018. Formulation and in vitro evaluation of a siRNA delivery nanosystem decorated with gH625 peptide for triple negative breast cancer theranosis. Eur. J. Pharm. Biopharm. 131, 99–108. https://doi.org/10.1016/j.ejpb.2018.07.024

Boussif, O., Lezoualc'ht, F., Zanta, M.A., Mergnyt, D., Schermant, D., Demeneixt, B., Behr, J.-P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine, Biochemistry.

Bruniaux, J., Djemaa, S. Ben, Hervé-Aubert, K., Marchais, H., Chourpa, I., David, S., Ben Djemaa, S., Hervé-Aubert, K., Marchais, H., Chourpa, I., David, S., 2017. Stealth magnetic nanocarriers of siRNA as platform for breast cancer theranostics. Int. J. Pharm. 532, 660–668. https://doi.org/10.1016/j.ijpharm.2017.05.022

Carthew, Richard W. and Sontheimer, E.J., Carthew, R.W., Sontheimer, E.J., 2009. Origins and Mechanisms of miRNAs and siRNAs. Cell 136, 642–655. https://doi.org/10.1016/j.cell.2009.01.035

Cavalieri, F., Beretta, G.L., Cui, J., Braunger, J.A., Yan, Y., Richardson, J.J., Tinelli, S., Folini, M., Zaffaroni, N., Caruso, F., 2015. Redox-sensitive PEG-polypeptide nanoporous particles for survivin silencing in prostate cancer cells. Biomacromolecules 16, 2168–2178. https://doi.org/10.1021/acs.biomac.5b00562

Cavallaro, G., Sardo, C., Craparo, E.F., Porsio, B., Giammona, G., 2017. Polymeric nanoparticles for siRNA delivery: Production and applications. Int. J. Pharm. 525, 313–333. https://doi.org/10.1016/J.IJPHARM.2017.04.008

Chen, M., Gao, S., Dong, M., Song, J., Yang, C., Howard, K.A., Al, C.E.T., 2012. Chitosan / siRNA Nanoparticles Encapsulated in PLGA Nanofibers for siRNA Delivery. ACS Nano 4835–4844.

Chen, Y., Gu, H., Zhang, D.S.-Z., Li, F., Liu, T., Xia, W., 2014. Highly effective inhibition of lung cancer growth and metastasis by systemic delivery of siRNA via multimodal mesoporous silica-based nanocarrier. Biomaterials 35, 10058–10069. https://doi.org/10.1016/J.BIOMATERIALS.2014.09.003

Chiper, M., Tounsi, N., Kole, R., Kichler, A., Zuber, G., 2017. Self-aggregating 1.8 kDa polyethylenimines with dissolution switch at endosomal acidic pH are delivery carriers for plasmid DNA, mRNA, siRNA and exon-skipping oligonucleotides. J. Control. Release 246, 60–70. https://doi.org/10.1016/J.JCONREL.2016.12.005

Conde, J., Dias, J.T., Grazú, V., Moros, M., Baptista, P. V, de la Fuente, J.M., 2014. Revisiting 30 years of biofunctionalization and surface chemistry of inorganic nanoparticles for nanomedicine. Front. Chem. 2, 1–27. https://doi.org/10.3389/fchem.2014.00048

Conner, S.D., Schmid, S.L., 2003. Regulated portals of entry into the cell. Nature 422, 37–44. https://doi.org/10.1038/nature01451

Corbet, C., Ragelle, H., Pourcelle, V., Vanvarenberg, K., Marchand-Brynaert, J., Préat, V., Feron, O., 2016. Delivery of siRNA targeting tumor metabolism using non-covalent PEGylated chitosan nanoparticles: Identification of an optimal combination of ligand structure, linker and grafting method. J. Control. Release 223, 53–63. https://doi.org/10.1016/j.jconrel.2015.12.020

Creusat, G., Rinaldi, A.S., Weiss, E., Elbaghdadi, R., Remy, J.S., Mulherkar, R., Zuber, G., 2010. Proton sponge trick for ph-sensitive disassembly of polyethylenimine-based sirna delivery systems. Bioconjug. Chem. 21, 994–1002. https://doi.org/10.1021/bc100010k

Creusat, G., Thomann, J.-S., Maglott, A., Pons, B., Dontenwill, M., Guérin, E., Frisch, B., Zuber, G., 2012. Pyridylthiourea-grafted polyethylenimine offers an effective assistance to siRNA-mediated gene silencing in vitro and in vivo. J. Control. Release 157, 418–426. https://doi.org/10.1016/J.JCONREL.2011.10.007

Creusat, G., Zuber, G., 2008. Self-Assembling Polyethylenimine Derivatives Mediate Efficient siRNA Delivery in Mammalian Cells. ChemBioChem 9, 2787–2789. https://doi.org/10.1002/cbic.200800540

David, S., Resnier, P., Guillot, A., Pitard, B., Benoit, J.-P., Passirani, C., 2012. siRNA LNCs – A novel platform of lipid nanocapsules for systemic siRNA administration. Eur. J. Pharm. Biopharm. 81, 448–452. https://doi.org/10.1016/J.EJPB.2012.02.010

Derfus, A.M., Chen, A.A., Min, D.-H., Ruoslahti, E., Bhatia, S.N., 2007. Targeted Quantum Dot Conjugates for siRNA Delivery. Bioconjug. Chem. 18, 1391–1396. https://doi.org/10.1021/bc060367e

Ding, Y., Wang, W., Feng, M., Wang, Y., Zhou, J., Ding, X., Zhou, X., Liu, C., Wang, R., Zhang, Q., 2012. A biomimetic nanovector-mediated targeted cholesterol-conjugated siRNA delivery for tumor gene therapy. Biomaterials 33, 8893–8905. https://doi.org/10.1016/j.biomaterials.2012.08.057

Ding, Y., Wang, Y., Zhou, J., Gu, X., Wang, W., Liu, C., Bao, X., Wang, C., Li, Y., Zhang, Q., 2014. Direct cytosolic siRNA delivery by reconstituted high density lipoprotein for target-specific therapy of tumor angiogenesis. Biomaterials 35, 7214–7227. https://doi.org/10.1016/j.biomaterials.2014.05.009

Dohmen, C., Fröhlich, T., Lächelt, U., Röhl, I., Vornlocher, H.-P., Hadwiger, P., Wagner, E., 2012. Defined Folate-PEG-siRNA Conjugates for Receptor-specific Gene Silencing. Mol. Ther. - Nucleic Acids 1, e7. https://doi.org/10.1038/MTNA.2011.10

Dominska, M., Dykxhoorn, D.M., 2010. Breaking down the barriers: siRNA delivery and endosome escape. J Cell Sci 123, 1183–1189. https://doi.org/10.1242/jcs.066399

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498. https://doi.org/10.1038/35078107

Fan, C., Wang, S., Hong, J.W., Bazan, G.C., Plaxco, K.W., Heeger, A.J., 2003. Beyond superquenching: Hyper-efficient energy transfer from conjugated polymers to gold nanoparticles. Proc. Natl. Acad. Sci. U. S. A. 100, 6297–6301. https://doi.org/10.1073/pnas.1132025100

Ferrari, M., Sun, T., Shen, H., Gilmore, J.H., 2012. Nanovector Delivery of siRNA for Cancer Therapy. Cancer Gene Ther. 19, 1883–1889. https://doi.org/10.1038/cgt.2012.22

Fischer, D., Bieber, T., Li, Y., Elsässer, H.P., Kissel, T., 1999. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: Effect of molecular weight on transfection efficiency and cytotoxicity. Pharm. Res. 16, 1273–1279. https://doi.org/10.1023/A:1014861900478

Foillard, S., Zuber, G., Doris, E., 2011. Polyethylenimine–carbon nanotube nanohybrids for siRNA-mediated gene silencing at cellular level. Nanoscale 3, 1461. https://doi.org/10.1039/c0nr01005g

Forster, J.C., Harriss-Phillips, W.M., Douglass, M.J., Bezak, E., 2017. A review of the development of tumor vasculature and its effects on the tumor microenvironment. Hypoxia (Auckland, N.Z.) 5, 21–32. https://doi.org/10.2147/HP.S133231

Fröhlich, T., Edinger, D., Kläger, R., Troiber, C., Salcher, E., Badgujar, N., Martin, I., Schaffert, D., Cengizeroglu, A., Hadwiger, P., Vornlocher, H.-P., Wagner, E., 2012. Structure–activity relationships of siRNA carriers based on sequence-defined oligo (ethane amino) amides. J. Control. Release 160, 532–541. https://doi.org/10.1016/J.JCONREL.2012.03.018

Galdiero, S., Falanga, A., Morelli, G., Galdiero, M., 2015. gH625: A milestone in understanding the many roles of membranotropic peptides. Biochim. Biophys. Acta - Biomembr. 1848, 16–25. https://doi.org/10.1016/j.bbamem.2014.10.006

Gary, D.J., Puri, N., Won, Y.-Y., 2007. Polymer-based siRNA delivery: Perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. J. Control. Release 121, 64–73. https://doi.org/10.1016/J.JCONREL.2007.05.021

Gavrilov, K., Saltzman, W.M., 2012. Therapeutic siRNA: principles, challenges, and strategies. Yale J. Biol. Med. 85, 187–200.

Gillies, R.J., Schornack, P.A., Secomb, T.W., Raghunand, N., 1999. Causes and effects of heterogeneous perfusion in tumors. Neoplasia 1, 197–207.

Goren, D., Horowitz, A.T., Zalipsky, S., Woodle, M.C., Yarden, Y., Gabizon, A., 1996. Targeting of stealth liposomes to erbB-2 (Her/2) receptor: in vitro and in vivo studies. Br. J. Cancer 74, 1749–1756.

Guruprasath, P., Kim, J., Gunassekaran, G.R., Chi, L., Kim, S.Y.S.S.-Y.H.S.-H., Park, R.-W.W., Kim, S.Y.S.S.-Y.H.S.-H., Baek, M.-C.C., Bae, S.M., Kim, S.Y.S.S.-Y.H.S.-H., Kim, D.-K.K., Park, I.-K.K., Kim, W.-J.J., Lee, B., 2017. Interleukin-4 receptor-targeted delivery of Bcl-xL siRNA sensitizes tumors to chemotherapy and inhibits tumor growth. Biomaterials 142, 101–111. https://doi.org/10.1016/j.biomaterials.2017.07.024

Haussecker, D., 2014. Current issues of RNAi therapeutics delivery and development. J. Control. Release 195, 49–54. https://doi.org/10.1016/j.jconrel.2014.07.056

Heldin, C.-H., Rubin, K., Pietras, K., Östman, A., 2004. High interstitial fluid pressure — an obstacle in cancer therapy. Nat. Rev. Cancer 4, 806–813. https://doi.org/10.1038/nrc1456

Huang, Y., Wang, X., Huang, W., Cheng, Q., Zheng, S., Guo, S., Cao, H., Liang, X.J., Du, Q., Liang, Z., 2015. Systemic Administration of siRNA via cRGD-containing Peptide. Sci. Rep. 5, 12458. https://doi.org/10.1038/srep12458

Huh, M.S., Lee, S.Y., Park, S., Lee, Seulki, Chung, H., Lee, Sojin, Choi, Y., Oh, Y.K., Park, J.H., Jeong, S.Y., Choi, K., Kim, K., Kwon, I.C., 2010. Tumor-homing glycol chitosan/polyethylenimine nanoparticles for the systemic delivery of siRNA in tumor-bearing mice. J. Control. Release 144, 134–143. https://doi.org/10.1016/j.jconrel.2010.02.023

Jabir, N.R., Tabrez, S., Ashraf, G.M., Shakil, S., Damanhouri, G.A., Kamal, M.A., 2012. Nanotechnology-based approaches in anticancer research. Int. J. Nanomedicine 7, 4391–4408. https://doi.org/10.2147/IJN.S33838

Jaganathan, H., Mitra, S., Srinivasan, S., Dave, B., Godin, B., 2014. Design and in vitro evaluation of layer by layer siRNA nanovectors targeting breast tumor initiating cells. PLoS One 9, e91986. https://doi.org/10.1371/journal.pone.0091986

Jain, R.K., Stylianopoulos, T., 2010. Delivering nanomedicine to solid tumors. Nat. Rev. Clin. Oncol. 7, 653–664. https://doi.org/10.1038/nrclinonc.2010.139

Jiang, T., Zhang, Z., Zhang, Y., Lv, H., Zhou, J., Li, C., Hou, L., Zhang, Q., 2012. Dual-functional liposomes based on pH-responsive cell-penetrating peptide and hyaluronic acid for tumor-targeted anticancer drug delivery. Biomaterials 33, 9246–9258. https://doi.org/10.1016/j.biomaterials.2012.09.027

Jonker, C., de Heus, C., Faber, L., ten Brink, C., Potze, L., Fermie, J., Liv, N., Klumperman, J., 2017. An adapted protocol to overcome endosomal damage caused by polyethylenimine (PEI) mediated transfections. Matters 3, e201711000012. https://doi.org/10.19185/matters.201711000012

Kaczmarek, J.C., Kowalski, P.S., Anderson, D.G., 2017. Advances in the delivery of RNA therapeutics: from concept to clinical reality. Genome Med. 9, 60. https://doi.org/10.1186/s13073-017-0450-0

Kang, L., Fan, B., Sun, P., Huang, W., Jin, M., Wang, Q., Gao, Z., 2016. An effective tumor-targeting strategy utilizing hypoxia-sensitive siRNA delivery system for improved anti-tumor outcome. Acta Biomater. 44, 341–354. https://doi.org/10.1016/J.ACTBIO.2016.08.029

Kievit, F.M., Veiseh, O., Fang, C., Bhattarai, N., Lee, D., Ellenbogen, R.G., Zhang, M., 2010. Chlorotoxin Labeled Magnetic Nanovectors for Targeted Gene Delivery to Glioma. ACS Nano 4, 4587–4594. https://doi.org/10.1021/nn1008512

Kim, E.J., Shim, G., Kim, K., Kwon, I.C., Oh, Y.K., Shim, C.K., 2009. Hyaluronic acid complexed to biodegradable poly L-arginine for targeted delivery of siRNAs. J. Gene Med. 11, 791–803. https://doi.org/10.1002/jgm.1352

Krivitsky, A., Polyak, D., Scomparin, A., Eliyahu, S., Ofek, P., Tiram, G., Kalinski, H., Avkin-Nachum, S., Feiner Gracia, N., Albertazzi, L., Satchi-Fainaro, R., 2018. Amphiphilic poly(α)glutamate polymeric micelles for systemic administration of siRNA to tumors. Nanomedicine Nanotechnology, Biol. Med. 14, 303–315. https://doi.org/10.1016/j.nano.2017.10.012

Landry, B., Aliabadi, H.M., Samuel, A., Gül-Uludağ, H., Jiang, X., Kutsch, O., Uludağ, H., 2012. Effective Non-Viral Delivery of siRNA to Acute Myeloid Leukemia Cells with Lipid-Substituted Polyethylenimines. PLoS One 7, e44197. https://doi.org/10.1371/journal.pone.0044197

Lee, D.-J., He, D., Kessel, E., Padari, K., Kempter, S., Lächelt, U., Rädler, J.O., Pooga, M., Wagner, E., 2016a. Tumoral gene silencing by receptor-targeted combinatorial siRNA polyplexes. J. Control. Release 244, 280–291. https://doi.org/10.1016/J.JCONREL.2016.06.011

Lee, D.-J., Kessel, E., Edinger, D., He, D., Klein, P.M., Voith von Voithenberg, L., Lamb, D.C., Lächelt, U., Lehto, T., Wagner, E., 2016b. Dual antitumoral potency of EG5 siRNA nanoplexes armed with cytotoxic bifunctional glutamyl-methotrexate targeting ligand. Biomaterials 77, 98–110. https://doi.org/10.1016/J.BIOMATERIALS.2015.11.004

Lee, J.E., Lee, K., Nam, J.A., Kim, A., Lee, S.Y., Lee, M.S., Kim, N.W., Yin, Y., Park, J.W., Park, S.Y., Jeong, J.H., 2018. Cellular Delivery of siRNA Using Poly(2-dimethylaminoethyl methacrylate)-Functionalized Graphene Oxide Nano-Wrap. Macromol. Res. 26, 1115–1122. https://doi.org/10.1007/s13233-019-7017-4

Liu, C., Liu, X., Rocchi, P., Qu, F., Iovanna, J.L., Peng, L., 2014. Arginine-terminated generation 4 PAMAM dendrimer as an effective nanovector for functional siRNA delivery in vitro and in vivo. Bioconjug. Chem. 25, 521–532. https://doi.org/10.1021/bc4005156

Liu, G., Xie, J., Zhang, F., Wang, Z.-Y., Luo, K., Zhu, L., Quan, Q.-M., Niu, G., Lee, S., Ai, H., Chen, X., 2011. N-Alkyl-PEI Functional Iron Oxide Nanocluster for Efficient siRNA Delivery**. Small 7, 2742–2749. https://doi.org/10.1002/smll.201100825

Liu, X., Peng, L., 2016. Dendrimer nanovectors for siRNA delivery. Methods Mol. Biol. 1364, 127–142. https://doi.org/10.1007/978-1-4939-3112-5_11

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., Darnell, J., 2000. Receptor-Mediated Endocytosis and the Sorting of Internalized Proteins.

Lu, A.-H., Salabas, E.L., Schüth, F., 2007. Magnetic nanoparticles: synthesis, protection, functionalization, and application. Angew. Chem. Int. Ed. Engl. 46, 1222–1244.

https://doi.org/10.1002/anie.200602866

Lv, H., Zhang, S., Wang, B., Cui, S., Yan, J., 2006. Toxicity of cationic lipids and cationic polymers in gene delivery. J. Control. Release Off. J. Control. Release Soc. 114, 100–109. https://doi.org/10.1016/j.jconrel.2006.04.014

Malhotra, M., Tomaro-Duchesneau, C., Saha, S., Prakash, S., 2013. Intranasal, siRNA Delivery to the Brain by TAT/MGF Tagged PEGylated Chitosan Nanoparticles. J. Pharm. 2013, 1–10. https://doi.org/10.1155/2013/812387

Marsh, M., McMahon, H.T., 1999. The structural era of endocytosis. Science 285, 215–220.

Menezes, D.E.L. de, Pilarski, L.M., Allen, T.M., 1998. In Vitro and in Vivo Targeting of Immunoliposomal Doxorubicin to Human B-Cell Lymphoma. Cancer Res. 58, 3320–3330.

Mi, R.P., Ki, O.H., In, K.H., Myung, H.C., Jae, W.N., Yun, J.C., Chong, S.C., 2005. Degradable polyethylenimine-alt-poly(ethylene glycol) copolymers as novel gene carriers. J. Control. Release 105, 367–380. https://doi.org/10.1016/j.jconrel.2005.04.008

Minakuchi, Y., Takeshita, F., Kosaka, N., Sasaki, H., Yamamoto, Y., Kouno, M., Honma, K., Nagahara, S., Hanai, K., Sano, A., Kato, T., Terada, M., Ochiya, T., 2004. Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing in vitro and in vivo. Nucleic Acids Res. 32, e109–e109. https://doi.org/10.1093/nar/gnh093

Miteva, M., Kirkbride, K.C., Kilchrist, K. V., Werfel, T.A., Li, H., Nelson, C.E., Gupta, M.K., Giorgio, T.D., Duvall, C.L., 2015. Tuning PEGylation of mixed micelles to overcome intracellular and systemic siRNA delivery barriers. Biomaterials 38, 97–107. https://doi.org/10.1016/j.biomaterials.2014.10.036

Mok, H., Veiseh, O., Fang, C., Kievit, F.M., Wang, F.Y., Park, J.O., Zhang, M., 2010. PH-sensitive siRNA nanovector for targeted gene silencing and cytotoxic effect in cancer cells. Mol. Pharm. 7, 1930–1939. https://doi.org/10.1021/mp100221h

Mokhtarieh, A.A., Lee, J., Kim, S., Lee, M.K., 2018. Preparation of siRNA encapsulated nanoliposomes suitable for siRNA delivery by simply discontinuous mixing. Biochim. Biophys. Acta - Biomembr. 1860, 1318–1325. https://doi.org/10.1016/J.BBAMEM.2018.02.027

Mu, P., Nagahara, S., Makita, N., Tarumi, Y., Kadomatsu, K., Takei, Y., 2009. Systemic delivery of siRNA specific to tumor mediated by atelocollagen: Combined therapy using siRNA targeting Bcl-xL and cisplatin against prostate cancer. Int. J. Cancer 125, 2978–2990. https://doi.org/10.1002/ijc.24382

Muratovska, A., Eccles, M.R., 2004. Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. FEBS Lett. 558, 63–68. https://doi.org/10.1016/S0014-5793(03)01505-9

Nguyen, J., Szoka, F.C., 2012. Nucleic acid delivery: The missing pieces of the puzzle? Acc. Chem. Res. 45, 1153–1162. https://doi.org/10.1021/ar3000162

Nikam, R.R., Gore, K.R., 2018. Journey of siRNA: Clinical Developments and Targeted Delivery. Nucleic Acid Ther. 28, 209–224. https://doi.org/10.1089/nat.2017.0715

Oh, B., Lee, M., 2014. Combined delivery of HMGB-1 box A peptide and S1PLyase siRNA in animal models of acute lung injury. J. Control. Release 175, 25–35. https://doi.org/10.1016/j.jconrel.2013.12.008

Ohsaki, M., Okuda, T., Wada, A., Hirayama, T., Niidome, T., Aoyagi, H., 2002. In vitro gene transfection using dendritic poly(L-lysine). Bioconjug. Chem. 13, 510–517. https://doi.org/10.1021/bc015525a

Owens III, D.E., Peppas, N.A., 2006. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int. J. Pharm. 307, 93–102. https://doi.org/10.1016/j.ijpharm.2005.10.010

Ozcan, G., Ozpolat, B., Coleman, R.L., Sood, A.K., Lopez-Berestein, G., Medicine, R., 2015. Preclinical and clinical development of siRNA-based therapeutics. Adv. Drug Deliv. Rev. 87, 108–119. https://doi.org/10.1016/j.addr.2015.01.007

Pan, J., Ruan, W., Qin, M., Long, Y., Wan, T., Yu, K., Zhai, Y., Wu, C., Xu, Y., 2018. Intradermal delivery of STAT3 siRNA to treat melanoma via dissolving microneedles. Sci. Rep. 8, 1117. https://doi.org/10.1038/s41598-018-19463-2

Pan, X., Thompson, R., Meng, X., Wu, D., Xu, L., 2010. Tumor-targeted RNA-interference: functional non-viral nanovectors. Am. J. Cancer Res. 1, 25–42.

Parmar, M.B., Bahadur, R., Lö, R., Uludağ, H., 2018. Additive Polyplexes to Undertake siRNA Therapy against CDC20 and Survivin in Breast Cancer Cells. https://doi.org/10.1021/acs.biomac.8b00918

Pärnaste, L., Arukuusk, P., Langel, K., Tenson, T., Langel, Ü., 2017. The Formation of Nanoparticles between Small Interfering RNA and Amphipathic Cell-Penetrating Peptides. Mol. Ther. Nucleic Acids 7, 1–10. https://doi.org/10.1016/j.omtn.2017.02.003

Perche, F., Biswas, S., Patel, N.R., Torchilin, V.P., 2016. Hypoxia-responsive copolymer for siRNA delivery, in: Methods in Molecular Biology. pp. 139–162. https://doi.org/10.1007/978-1-4939-3148-4_12

Perche, F., Biswas, S., Wang, T., Zhu, L., Torchilin, V.P., 2014. Hypoxia-targeted siRNA delivery. Angew. Chemie - Int. Ed. 53, 3362–3366. https://doi.org/10.1002/anie.201308368

Pittella, F., Zhang, M., Lee, Y., Kim, H.J., Tockary, T., Osada, K., Ishii, T., Miyata, K., Nishiyama, N., Kataoka, K., 2011. Enhanced endosomal escape of siRNA-incorporating hybrid nanoparticles from calcium phosphate and PEG-block charge-conversional polymer for efficient gene knockdown with negligible cytotoxicity. Biomaterials 32, 3106–3114. https://doi.org/10.1016/j.biomaterials.2010.12.057

Prokop, A., Davidson, J.M.J., 2008. Nanovehicular Intracellular Delivery Systems. J. Pharm. Sci. 97, 3518–3590. https://doi.org/10.1002/jps.21270

Ragelle, H., Colombo, S., Pourcelle, V., Vanvarenberg, K., Vandermeulen, G., Bouzin, C., Marchand-Brynaert, J., Feron, O., Foged, C., Préat, V., 2015. Intracellular siRNA delivery dynamics of integrin-targeted, PEGylated chitosan-poly(ethylene imine) hybrid nanoparticles: A mechanistic insight. J. Control. Release 211, 1–9. https://doi.org/10.1016/j.jconrel.2015.05.274

Ratcliff, F., Harrison, B.D., Baulcombe, D.C., 1997. A Similarity Between Viral Defense and Gene Silencing in Plants. Science (80-.). 276, 1558–1560. https://doi.org/10.1126/science.276.5318.1558

Rehman, Z. ur, Hoekstra, D., Zuhorn, I.S., 2013. Mechanism of polyplex- and lipoplex-mediated delivery of nucleic acids: Real-time visualization of transient membrane destabilization without endosomal lysis. ACS Nano 7, 3767–3777. https://doi.org/10.1021/nn3049494

Reischl, D., Zimmer, A., 2009. Drug delivery of siRNA therapeutics: potentials and limits of nanosystems. Nanomedicine Nanotechnology, Biol. Med. 5, 8–20. https://doi.org/10.1016/J.NANO.2008.06.001

Resnier, P., Montier, T., Mathieu, V., Benoit, J.-P.P., Passirani, C., 2013. A review of the current status of siRNA nanomedicines in the treatment of cancer. Biomaterials 34, 6429–6443. https://doi.org/10.1016/j.biomaterials.2013.04.060

Reynolds, A., Anderson, E.M., Vermeulen, A., Fedorov, Y., Robinson, K., Leake, D., Karpilow, J., Marshall, W.S., Khvorova, A., 2006. Induction of the interferon response by siRNA is cell type-and duplex length-dependent. RNA 12, 988–93. https://doi.org/10.1261/rna.2340906

Richards Grayson, A.C., Doody, A.M., Putnam, D., 2006. Biophysical and Structural Characterization of Polyethylenimine-Mediated siRNA Delivery in Vitro. Pharm. Res. 23, 1868–1876. https://doi.org/10.1007/s11095-006-9009-2

Rizk, M., Tüzmen, Ş., 2017. Update on the clinical utility of an RNA interference-based treatment: focus on Patisiran. Pharmgenomics. Pers. Med. 10, 267–278. https://doi.org/10.2147/PGPM.S87945

Rosi, N.L., Giljohann, D.A., Thaxton, C.S., Lytton-Jean, A.K.R., Han, M.S., Mirkin, C.A., 2006. Oligonucleotide-modified gold nanoparticles for intracellular gene regulation. Science 312, 1027–1030. https://doi.org/10.1126/science.1125559

Rothbard, J.B., Jessop, T.C., Lewis, R.S., Murray, B.A., Wender, P.A., 2004. Role of Membrane Potential and Hydrogen Bonding in the Mechanism of Translocation of Guanidinium-Rich Peptides into Cells. J. Am. Chem. Soc. 126, 9506–9507. https://doi.org/10.1021/ja0482536

Scherer, F., Anton, M., Schillinger, U., Henke, J., Bergemann, C., Krüger, A., Gänsbacher, B., Plank, C., 2002. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. Gene Ther. 9, 102–109. https://doi.org/10.1038/sj.gt.3301624

Schmohl, K.A., Gupta, A., Grünwald, G.K., Trajkovic-Arsic, M., Klutz, K., Braren, R., Schwaiger, M., Nelson, P.J., Ogris, M., Wagner, E., Siveke, J.T., Spitzweg, C., 2017. Imaging and targeted therapy of pancreatic ductal adenocarcinoma using the theranostic sodium iodide symporter (NIS) gene. Oncotarget 8, 33393–33404. https://doi.org/10.18632/oncotarget.16499

Schubert, U.S., Traeger, A., Bus, T., 2018. The great escape: How cationic polyplexes overcome the endosomal barrier. J. Mater. Chem. B 6, 6904–6918. https://doi.org/10.1039/C8TB00967H

Shakiba, A., Zenasni, O., Marquez, M.D., Lee, T.R., 2017. Advanced drug delivery via self-assembled monolayer-coated nanoparticles. AIMS Bioeng. 4, 275–299. https://doi.org/10.3934/bioeng.2017.2.275

Shim, M.S., Kwon, Y.J., 2010. Efficient and targeted delivery of siRNA in vivo. FEBS J. 277, 4814–4827. https://doi.org/10.1111/j.1742-4658.2010.07904.x

Song, E., Lee, S.-K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., Lieberman, J., 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. 9, 347–351. https://doi.org/10.1038/nm828

Song, W.-J., Du, J.-Z., Sun, T.-M., Zhang, P.-Z., Wang, J., 2010. Gold Nanoparticles Capped with Polyethyleneimine for Enhanced siRNA Delivery. Small 6, 239–246. https://doi.org/10.1002/smll.200901513

Sosnovik, D.E., Nahrendorf, M., Weissleder, R., 2008. Magnetic nanoparticles for MR imaging: agents, techniques and cardiovascular applications. Basic Res. Cardiol. 103, 122–130. https://doi.org/10.1007/s00395-008-0710-7

Sun, C.-Y.Y., Shen, S., Xu, C.-F.F., Li, H.-J.J., Liu, Y., Cao, Z.-T.T., Yang, X.-Z.Z., Xia, J.-X.X., Wang, J., 2015. Tumor Acidity-Sensitive Polymeric Vector for Active Targeted siRNA Delivery. J. Am. Chem. Soc. 137, 15217–15224. https://doi.org/10.1021/jacs.5b09602

Sun, C., Lee, J.S.H., Zhang, M., 2008. Magnetic Nanoparticles in MR Imaging and Drug Delivery. Adv. Drug Deliv. Rev. 60, 1252–1265. https://doi.org/10.1016/j.addr.2008.03.018

Sun, P., Huang, W., Jin, M., Wang, Q., Fan, B., Kang, L., Gao, Z., 2016. Chitosan-based nanoparticles for survivin targeted siRNA delivery in breast tumor therapy and preventing its metastasis. Int. J. Nanomedicine 11, 4931–4945. https://doi.org/10.2147/IJN.S105427

Tabernero, J., Shapiro, G.I., LoRusso, P.M., Cervantes, A., Schwartz, G.K., Weiss, G.J., Paz-Ares, L., Cho, D.C., Infante, J.R., Alsina, M., Gounder, M.M., Falzone, R., Harrop, J., White, A.C.S., Toudjarska, I., Bumcrot, D., Meyers, R.E., Hinkle, G., Svrzikapa, N., Hutabarat, R.M., Clausen, V.A., Cehelsky, J., Nochur, S. V., Gamba-Vitalo, C., Vaishnaw, A.K., Sah, D.W.Y., Gollob, J.A., Burris, H.A., 2013. First-in-Humans Trial of an RNA Interference Therapeutic Targeting VEGF and KSP in Cancer Patients with Liver Involvement. Cancer Discov. 3, 406–417. https://doi.org/10.1158/2159-8290.CD-12-0429

Takeshita, F., Minakuchi, Y., Nagahara, S., Honma, K., Sasaki, H., Hirai, K., Teratani, T., Namatame, N., Yamamoto, Y., Hanai, K., Kato, T., Sano, A., Ochiya, T., 2005. Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo. Proc. Natl. Acad. Sci. U. S. A. 102, 12177–82. https://doi.org/10.1073/pnas.0501753102

Tan, S.J., Kiatwuthinon, P., Roh, Y.H., Kahn, J.S., Luo, D., 2011. Engineering Nanocarriers for siRNA Delivery. Small 7, 841–856. https://doi.org/10.1002/smll.201001389

Tatiparti, K., Sau, S., Kashaw, S.K., Iyer, A.K., 2017. siRNA Delivery Strategies: A Comprehensive Review of Recent Developments. Nanomater. (Basel, Switzerland) 7. https://doi.org/10.3390/nano7040077

Tian, H., Xiong, W., Wei, J., Wang, Y., Chen, X., Jing, X., Zhu, Q., 2007. Gene transfection of hyperbranched PEI grafted by hydrophobic amino acid segment PBLG. Biomaterials 28, 2899–2907. https://doi.org/10.1016/j.biomaterials.2007.02.027

Trützschler, A.K., Bus, T., Reifarth, M., Brendel, J.C., Hoeppener, S., Traeger, A., Schubert, U.S., 2018. Beyond Gene Transfection with Methacrylate-Based Polyplexes - The Influence of the Amino Substitution Pattern. Bioconjug. Chem. 29, 2181–2194. https://doi.org/10.1021/acs.bioconjchem.8b00074

Tuma, P.L., Hubbard, A.L., 2003. Transcytosis: Crossing Cellular Barriers. Physiol. Rev. 83, 871–932. https://doi.org/10.1152/physrev.00001.2003

Veiseh, O., Kievit, F.M., Ellenbogen, R.G., Zhang, M., 2011a. Cancer Cell Invasion: Treatment and Monitoring Opportunities in Nanomedicine. Adv. Drug Deliv. Rev., Target Cell Movement in Tumor and Cardiovascular Diseases 63, 582–596. https://doi.org/10.1016/j.addr.2011.01.010

Veiseh, O., Kievit, F.M., Fang, C., Mu, N., Jana, S., Leung, M.C., Mok, H., Ellenbogen, R.G., Park, J.O., Zhang, M., 2010. Chlorotoxin bound magnetic nanovector tailored for cancer cell targeting, imaging, and siRNA delivery. Biomaterials 31, 8032–8042. https://doi.org/10.1016/j.biomaterials.2010.07.016

Veiseh, O., Kievit, F.M., Mok, H., Ayesh, J., Clark, C., Fang, C., Leung, M., Arami, H., Park, J.O., Zhang, M., 2011b. Cell transcytosing poly-arginine coated magnetic nanovector for safe and effective siRNA delivery. Biomaterials 32, 5717–5725. https://doi.org/10.1016/j.biomaterials.2011.04.039

Veiseh, O., Sun, C., Fang, C., Bhattarai, N., Gunn, J., Kievit, F., Du, K., Pullar, B., Lee, D., Ellenbogen, R.G., Olson, J., Zhang, M., 2009. Specific targeting of brain tumors with an optical/magnetic resonance imaging nanoprobe across the blood-brain barrier. Cancer Res. 69, 6200–7. https://doi.org/10.1158/0008-5472.CAN-09-1157

Venditti, I., 2017. Morphologies and functionalities of polymeric nanocarriers as chemical tools for drug delivery: A review. J. King Saud Univ. - Sci. https://doi.org/10.1016/j.jksus.2017.10.004

Videira, M., Arranja, A., Rafael, D., Gaspar, R., 2014. Preclinical development of siRNA therapeutics: Towards the match between fundamental science and engineered systems. Nanomedicine Nanotechnology, Biol. Med. 10, 689–702. https://doi.org/10.1016/j.nano.2013.11.018

Wang, F., Wang, Y., Zhang, X., Zhang, W., Guo, S., Jin, F., 2014. Recent progress of cell-penetrating peptides as new carriers for intracellular cargo delivery. J. Control. Release 174, 126–136. https://doi.org/10.1016/j.jconrel.2013.11.020

Wang, J., Lu, Z., Wientjes, M.G., Au, J.L.-S., 2010. Delivery of siRNA Therapeutics: Barriers and Carriers. AAPS J. 12, 492–503. https://doi.org/10.1208/s12248-010-9210-4

Wang, T., Shigdar, S., Shamaileh, H. Al, Gantier, M.P., Yin, W., Xiang, D., Wang, L., Zhou, S.-F., Hou, Y., Wang, P., Zhang, W., Pu, C., Duan, W., 2017. Challenges and opportunities for siRNA-based cancer treatment. Cancer Lett. 387, 77–83. https://doi.org/10.1016/J.CANLET.2016.03.045

Wang, X.-L., Xu, R., Wu, X., Gillespie, D., Jensen, R., Lu, Z.-R., 2009. Targeted Systemic Delivery of a Therapeutic siRNA with a Multifunctional Carrier Controls Tumor Proliferation in Mice. Mol. Pharm. 6, 738–746. https://doi.org/10.1021/mp800192d

Werfel, T.A., Jackson, M.A., Kavanaugh, T.E., Kirkbride, K.C., Miteva, M., Giorgio, T.D., Duvall, C., 2017. Combinatorial optimization of PEG architecture and hydrophobic content improves ternary siRNA polyplex stability, pharmacokinetics, and potency in vivo. J. Control. Release 255, 12–26. https://doi.org/10.1016/j.jconrel.2017.03.389

Xie, Y., Qiao, H., Su, Z., Chen, M., Ping, Q., Sun, M., 2014. PEGylated carboxymethyl chitosan/calcium phosphate hybrid anionic nanoparticles mediated hTERT siRNA delivery for anticancer therapy. Biomaterials 35, 7978–7991. https://doi.org/10.1016/j.biomaterials.2014.05.068

Yin, T., Liu, J., Zhao, Z., Dong, L., Cai, H., Yin, L., Zhou, J., Huo, M., 2016. Smart nanoparticles with a detachable outer shell for maximized synergistic antitumor efficacy of therapeutics with varying physicochemical properties. J. Control. Release 243, 54–68. https://doi.org/10.1016/j.jconrel.2016.09.036

Zhang, W., Müller, K., Kessel, E., Reinhard, S., He, D., Klein, P.M., Höhn, M., Rödl, W., Kempter, S., Wagner, E., 2016. Targeted siRNA Delivery Using a Lipo-Oligoaminoamide Nanocore with an Influenza Peptide and Transferrin Shell. Adv. Healthc. Mater. 5, 1493–1504. https://doi.org/10.1002/adhm.201600057

Zhu, H., Zhang, S., Ling, Y., Meng, G., Yang, Y., Zhang, W., 2015. PH-responsive hybrid quantum dots for targeting hypoxic tumor siRNA delivery. J. Control. Release 220, 529–544. https://doi.org/10.1016/j.jconrel.2015.11.017

Table 1. Examples of the most used polymers in electrostatically assembled polymer-based siRNA nanovectors

Polymer	Abbreviation	Charge	MW	References
Chitosan	CS	Cationic	110 – 250 KDa	(Chen et al., 2012; Huh et al., 2010; Sun et al., 2016; Veiseh et al., 2010; Xie et al., 2014)
Atelocollagen	ATCOL	Cationic	300 KDa	(Minakuchi et al., 2004; Mu et al., 2009)
Polyethylenimine	PEI	Cationic	1.2 – 25 KDa	(Huh et al., 2010; Liu et al., 2011; Mok et al., 2010; Veiseh et al., 2011b, 2010)
Poly-arginine/poly-L-arginine	pArg/PLR	Cationic	10 – 70 KDa	(Ben Djemaa et al., 2018; Bruniaux et al., 2017; Kim et al., 2009; Veiseh et al., 2011b)
Poly-lysine/poly-L-lysine	pLys /PLL	Cationic	10 – 70 KDa	(Cavalieri et al., 2015; Jaganathan et al., 2014; Veiseh et al., 2011b)
Poly-alpha-glutamate	PGA	Cationic	7 KDa	(Krivitsky et al., 2018)
Poly-amidoamine	PAMAM	Cationic	20 – 80 KDa	(Liu et al., 2014; Liu and Peng, 2016)
Polyaspartamide-1,2-diaminoethane	PAsp(DET)	Cationic		(Pittella et al., 2011)
Poly(dimethylaminoethyl methacrylate)	pDMAEMA	Cationic	12 KDa	(Lee et al., 2018; Miteva et al., 2015)

Poly[dimethylaminoethyl methacrylate-b-(dimethylaminoethyl methacrylate-co-propylacrylic acidco-butyl methacrylate)]	pD-b-pDPB	Cationic	32 KDa	(Miteva et al., 2015)
Hyaluronic acid	HA	Anionic	19 – 50 KDa	(Kim et al., 2009; Yin et al., 2016)
Poly-D,L-lactic-co-glycolic acid	PLGA	Anionic	66 – 107 KDA	(Chen et al., 2012)
Polyethylene glycol	PEG	Neutral	2 – 12 KDa	(Cavalieri et al., 2015; Pittella et al., 2011; Sun et al., 2016; Veiseh et al., 2011b, 2010; Werfel et al., 2017)

Table 2. Examples of the most used peptides for the functionalization of electrostatically assembled polymer-based siRNA nanovectors

Peptide	Abbreviation	Origin	Family	Sequence	Target	Reference
Oligo-arginine	R8, R9, R11, …	Synthetic peptide	Cell-penetrating peptide	Rn (n = 8, 9, 11 …)	Not identified	(Liu et al., 2014)
Trans-activated transcription	TAT	Protein transduction domain of human immunodeficiency virus type 1	Cell-penetrating peptide	GRKKRRQRRRPPQ	No data	(Malhotra et al., 2013)
Penetratin	P	Homeodomain of the Drosophila homeoprotein Antennapedia	Cell-penetrating peptide	CRQIKIWFQNRRMKWKK	No data	(Muratovska and Eccles, 2004)

gH625	gH625	Glycoprotein H of Herpes simplex virus type 1	Cell-penetrating peptide	HGLASTLTRWAHYNALIRAF	Not identified	(Ben Djemaa et al., 2018)
Transportan	TP 10	Galanin and mastoparan	Cell-penetrating peptide	GWTLNSAGYLLGKINLKALAALAKKIL	No data	(Pärnaste et al., 2017)
Chlorotoxin	CTX	Scorpion-derived peptide	Tumor-targeting peptide	MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR	affinity to the vast majority of brain tumors, prostate, skin and colorectal cancers	(Mok et al., 2010; Veiseh et al., 2010)
Arginine-glycine-aspartate	RGD	Synthetic	Receptor-recognition	RGD	Tumor endothelial cells	(Huang et al., 2015; Ragelle et

			motif			al., 2015; Wang et al., 2009)
IL-4 receptor-binding peptide	IL4RPep-1	Synthetic	Peptide	CRKRLDRNC	IL-4 receptor	(Guruprasath et al., 2017)
RRRVVVVVV	R3V6	Synthetic	Cell-penetrating peptide	RRRVVVVVV	Not identified	(Oh and Lee, 2014)
Bombesin	BN	Skin of an European frog	Peptide	QRLGNQWAVGHLM	Gastrin-releasing peptide receptors	(Wang et al., 2009)

Table 3. Internalization pathways and endosomal escape studies of electrostatically assembled polymer-based siRNA nanovectors

Entry pathway	**Nanovector**	**Techniques used for endosomal**	**Main result**	**Ref**

		studies		
Receptor-mediated endocytosis	NP-siRNA-CTX	Fluorescence microscopy Endosomal integrity assay: calcein	Nanovectors are able to escape endosomes	(Veiseh et al., 2010)
	HA-^{PTX}PSR$_{siRNA}$	Confocal microscopy	Decrease of the colocalization of nanovectors with lysotracker after 24 h compared to 2 h	(Yin et al., 2016)
	IL-4R-targeted BPEI-SPION/siRNA	Confocal microscopy	Nanovectors are detected in early endosomes. After 24 h nanovectors are detected in late endosomes, lysosomes and cytosol	(Guruprasath et al., 2017)
	9R/DG-QDs	Confocal microscopy	No colocalization between nanovectors and lysosomes after 28 h of cell treatment and nanovectors are localized in the cytosol	(Zhu et al., 2015)
Macropinocytosis	PEG-CMCS/CaP hybrid anionic nanoparticles	Confocal microscopy	Nanovectors escape endosomes and pass in cytosol	(Xie et al., 2014)
Non-specified endocytosis	PEG-polyanion/siRNA/CaP hybrid nanoparticles	Confocal microscopy	Nanovectors escape endosomes and pass in cytosol	(Pittella et al., 2011)
	Mixed micelles	Confocal microscopy	Low colocalization with lysotracker	(Miteva et

			al., 2015)
NPEG−PLLs	Flow cytometry Confocal microscopy	Decrease of the colocalization of nanovectors with lysosomes after 24 h	(Cavalieri et al., 2015)
ternary siRNA polyplexes	Confocal microscopy	Low colocalization with endosomes and cytosolic dispersion	(Werfel et al., 2017)

NP: nanoparticles; **CTX**: chlorotoxin; **HA**: hyaluronic acid; **PTX**: Paclitaxel; **PSR**: Octyl modified polyethyleneimine containing disulfide linkages; **IL-4R**: interleukin 4 receptor; **BPEI**: branched PEI; **SPION**: superparamagnetic iron oxide nanoparticles; **DG**: 2-deoxyglucose; **QDs**: quantum dots; **PEG**: polyethylene glycol; **CMCS**: carboxymethyl chitosan; **CaP**: calcium phosphate; **PLL**: poly-L-lysin

Table 4. Electrostatically assembled polymer-based siRNA nanovectors studied *in vitro*

Nanovector	**Composition**	**Cells**	**Target gene**	**siRNA concentration**	**Treatment time (h)**	**Silencing efficiency (%)**	**Reference**
ternary siRNA polyplexes	DMAEMA, BMA, PEG, siRNA	MDA-MB-231	Luciferase	100 nM	24	85	(Werfel et al., 2017)

		NIH3T3 MSC					
PEG-polyanion/siRNA/CaP hybrid nanoparticles	CaP, PEG, CCP, siRNA	ApanC-1	Luciferase	60 nM	3	82	(Pittella et al., 2011)
Mixed micelles	PEG-b-pDPB, pD-b-pDPB, siRNA	MDA-MB-231	Luciferase	100 nM	24	80	(Miteva et al., 2015)
Alkyl-PEI2k-IO/siRNA	Iron oxide, Alkyl-PEI, siRNA	4T1	Luciferase	6 pM	3	80	(Liu et al., 2011)
RGDp NP	Integrin-arginine-glycine-aspartate, PEG, chitosan, PEI, siRNA	H1299	GFP	100 nM	4	80	(Ragelle et al., 2015)
PEG-CMCS/CaP hybrid anionic nanoparticles	PEG, carboxymethyl chitosan, calcium, phosphate, siRNA	Hep G2	Luciferase hTERT	100 nM	48	79 60	(Xie et al., 2014)
RGDp R1 NP	PEG, chitosan, RGDp, siRNA	H1299 SiHa	GFP ASCT2	100 nM	6	70	(Corbet et al., 2016)

			MCT1				
NP-pArg-siRNA	SPION, PEG, pArg, siRNA	C6 MCF7 TC2	GFP	No data	8	68	(Veiseh et al., 2011b)
NP-siRNA-CTX	SPION, PEG, chitosan, PEI, chlorotoxin, siRNA	C6	GFP	225 nM	2	62	(Veiseh et al., 2010)
NPEG–PLLs	NPEG-PLL, siRNA	PC-3	Survivine	31 nM	120	60	(Cavalieri et al., 2015)
HA-^{PTX}PSR$_{siRNA}$	PEI, hyaluronic acid, siRNA (HA-PTX_PSR-siRNA)	A549	PIK1	80 nM	6	60	(Yin et al., 2016)
9R/DG-QDs	2-deoxyglucose (DG), PEG, lipoic acid-lysine- 9-poly-d-arginine (LA–Lys–9R), QDs, siRNA	Hep G2	GLUT1 HIF_1 α	50 nM	2	60	(Zhu et al., 2015)
NP-pLys-siRNA	SPION, PEG, pLys, siRNA	C6 MCF7	GFP	No data	8	39	(Veiseh et al., 2011b)

		TC2					
NP-PEI-siRNA	SPION, PEG, PEI, siRNA	C6 MCF7 TC2	GFP	No data	8	32	(Veiseh et al., 2011b)

DMAEMA: [2-(dimethylamino) ethyl methacrylate]; **BMA**: butyl methacrylate; **PEG**: polyethylene glycol; **CaP**: calcium phosphate; **CCP**: charge-conversional polymer; **b-pDPB**: b-(dimethylaminoethyl methacrylate-co-propylacrylic acid-co-butyl methacrylate); **pD-b-pDPB:** poly[dimethylaminoethyl methacrylate-b-(dimethylaminoethyl methacrylate-co-propylacrylic acidco-butyl methacrylate)]; **IO**: iron oxide; **PEI**: polyethyleneimine; **RGDp**: arginine-glycine-aspartate peptide; **NP**: nanoparticles; **CMCS**: carboxymethyl chitosan; **hTERT**: human telomerase reverse transcriptase; **pArg**: polyarginine; **SPION**: superparamagnetic iron oxide nanoparticles; **CTX**: chlorotoxin; **PLL**: poly-L-lysin; **HA**: hyaluronic acid; **PTX**: Paclitaxel; **PSR**: Octyl modified polyethyleneimine containing disulfide linkages; **DG**: 2-deoxyglucose; **QDs**: quantum dots; **pLys**: polylysin

Table 5. Electrostatically assembled polymer-based siRNA nanovectors studied *in vivo*

Nanovector	**Composition**	**Model**	**Target gene**	**siRNA dose**	**Administration protocol (number of injection)**	**Administration route**	**Silencing efficiency (%)**	**Reference**
HA-^{PTX}PSR$_{siRNA}$	PEI, hyaluronic acid, siRNA (HA-PTX_PSR-siRNA)	4T1-Fluc cells BALB/c nude mice	PIK1	0.5 mg/kg	3	Intravenously	60	(Yin et al., 2016)
Alkyl-PEI2k-IO/siRNA	Iron oxide, Alkyl-PEI, siRNA	A549 cells Athymic nude mice	Luciferase	250 pg/kg	1 day/2, for 34 days	Intratumorally	60	(Liu et al., 2011)
ternary siRNA polyplexes	DMAEMA, BMA, PEG, siRNA	L231 cells Athymic nude mice	Luciferase	1 mg/kg	2	Intravenously	59	(Werfel et al., 2017)

PEG-CMCS/CaP hybrid anionic nanoparticles	PEG, carboxymethyl chitosan, calcium, phosphate, siRNA	HepG2 cells BALB/c nude mice	Luciferase hTERT	1.2 mg/kg	2	Intravenously	57	(Xie et al., 2014)
IL-4R-targeted BPEI-SPION/siRNA	SPION, PEI, IL4RPep1, siRNA	MDA-MB231 cells BALB/c nude mice	Bcl-xL	0.15 mg/kg	3/ week for 4 weeks	Intravenously	40	(Guruprasath et al., 2017)
RGDp R1 NP	PEG, chitosan, RGDp, siRNA	SiHa cells NMRI nude mice	ASCT2 MCT1	2 mg/kg	2/ week for 2 weeks	Intravenously Peritumoral	60	(Corbet et al., 2016)
9R/DG-QDs	2-deoxyglucose (DG), PEG, lipoic acid- lysine- 9-poly-d-arginine (LA–Lys–	HepG2 cells Kunming mice	HIF_1α	3 mg/kg	8 (1 day /2)	Intravenously	No data	(Zhu et al., 2015)

	9R), QDs, siRNA

HA: hyaluronic acid; **PTX**: Paclitaxel; **PSR**: Octyl modified polyethyleneimine containing disulfide linkages; **PEI**: polyethyleneimine; **IO**: iron oxide; **DMAEMA**: [2-(dimethylamino) ethyl methacrylate]; **BMA**: butyl methacrylate; **PEG**: polyethylene glycol; **CMCS**: carboxymethyl chitosan; **CaP**: calcium phosphate; **hTERT**: human telomerase reverse transcriptase; **IL-4R**: interleukin 4 receptor; **SPION**: superparamagnetic iron oxide nanoparticles; **BPEI**: branched PEI; **RGDp**: arginine-glycine-aspartate peptide; **DG**: 2-deoxyglucose; **QDs**: quantum dots

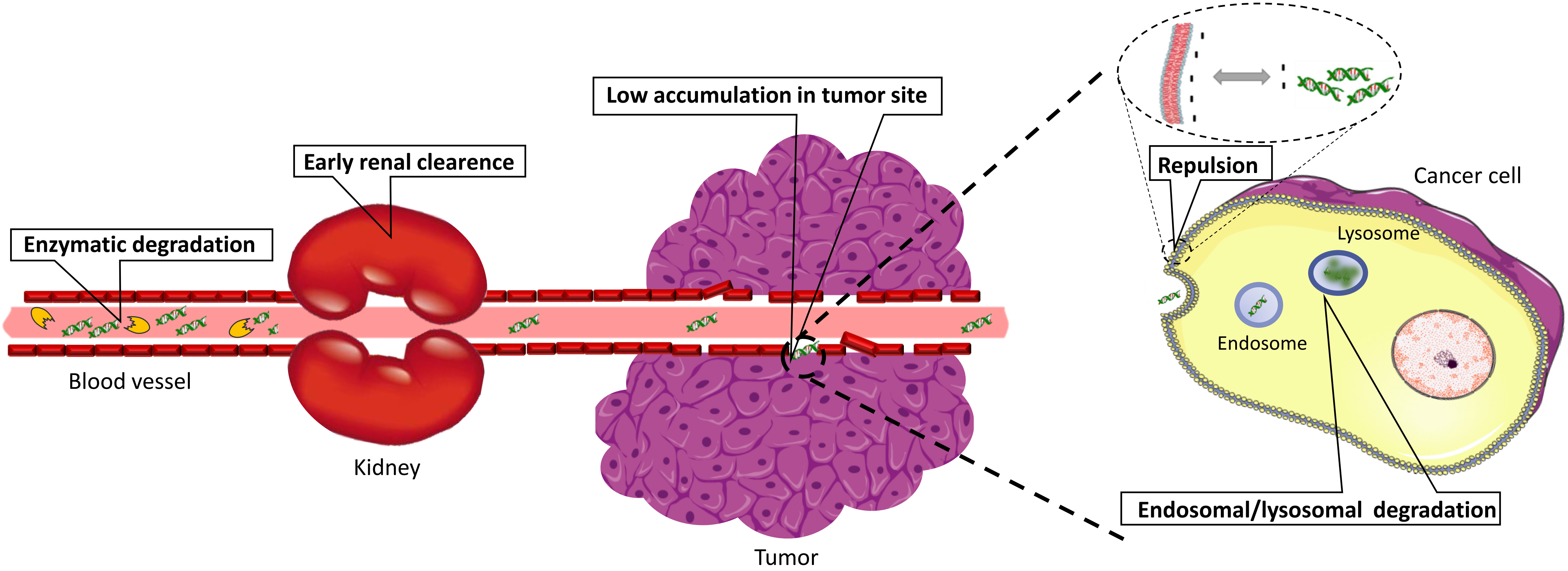
Figure captions

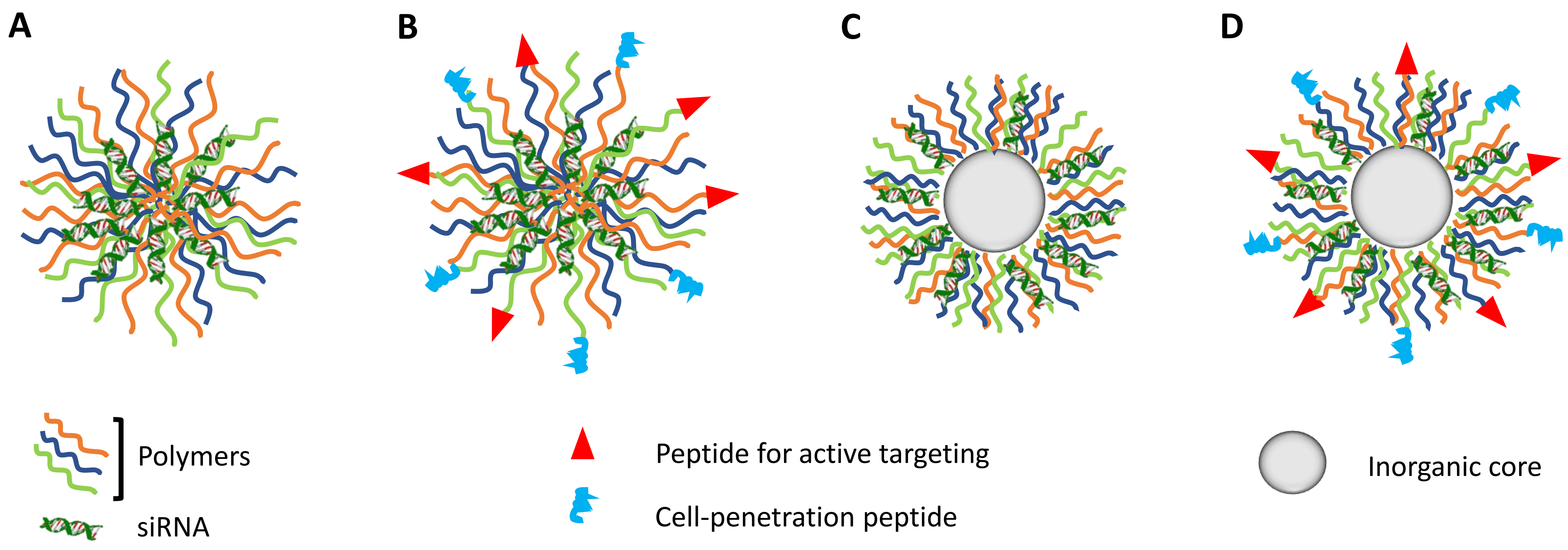
Figure 1. Illustration of extra- and intra-cellular biological barriers for siRNA-based cancer therapy. Extracellular barriers: enzymatic degradation in the blood, early elimination by the kidney, low accumulation in the tumor site, and repulsion at the surface of the cell membrane. Intracellular barriers: endosomal entrapment and endo-lysosomal degradation.

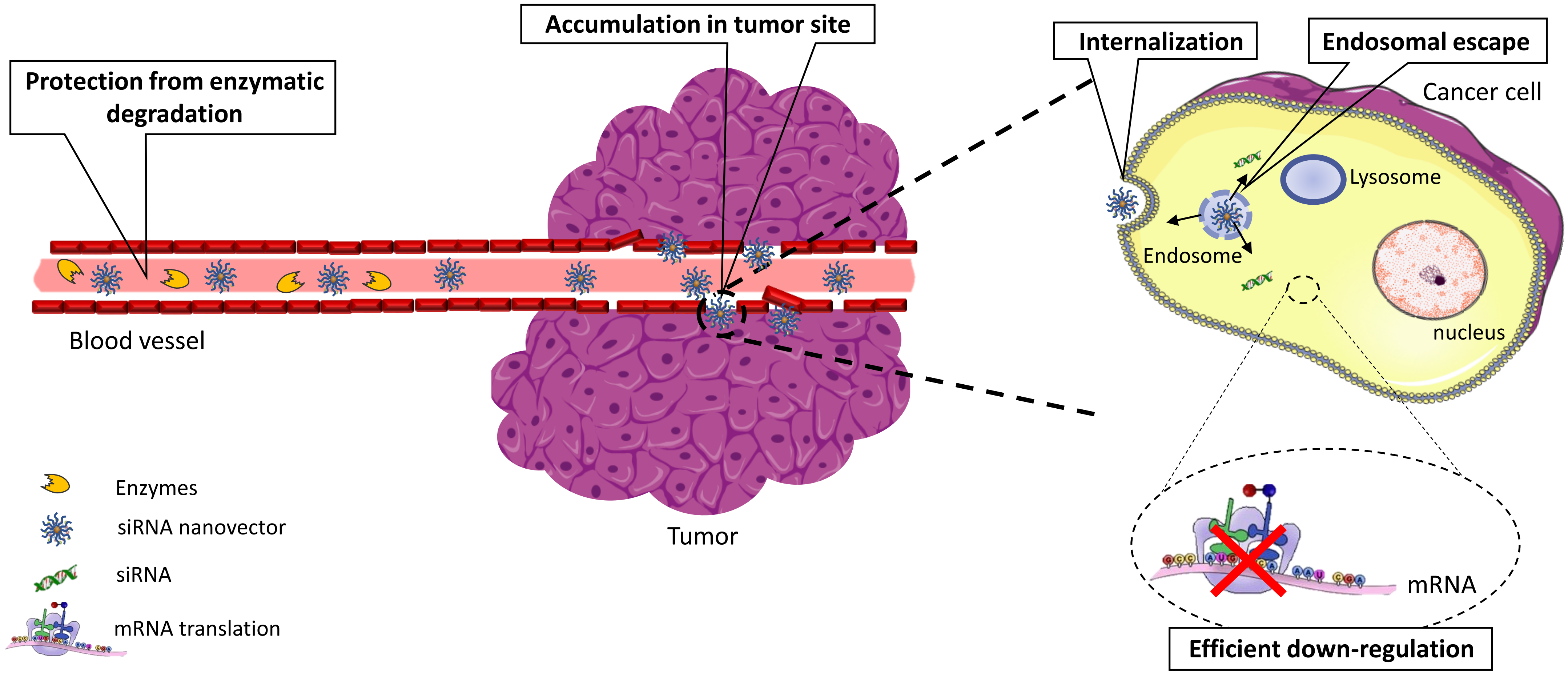
Figure 2. Schematic presentation of electrostatic assembled polymer-based siRNA nanovectors (EPSN): (A) containing only polymers and siRNA, (B) decorated with peptides, (C) containing an inorganic core, and (D) containing an inorganic core and decorated with peptides.

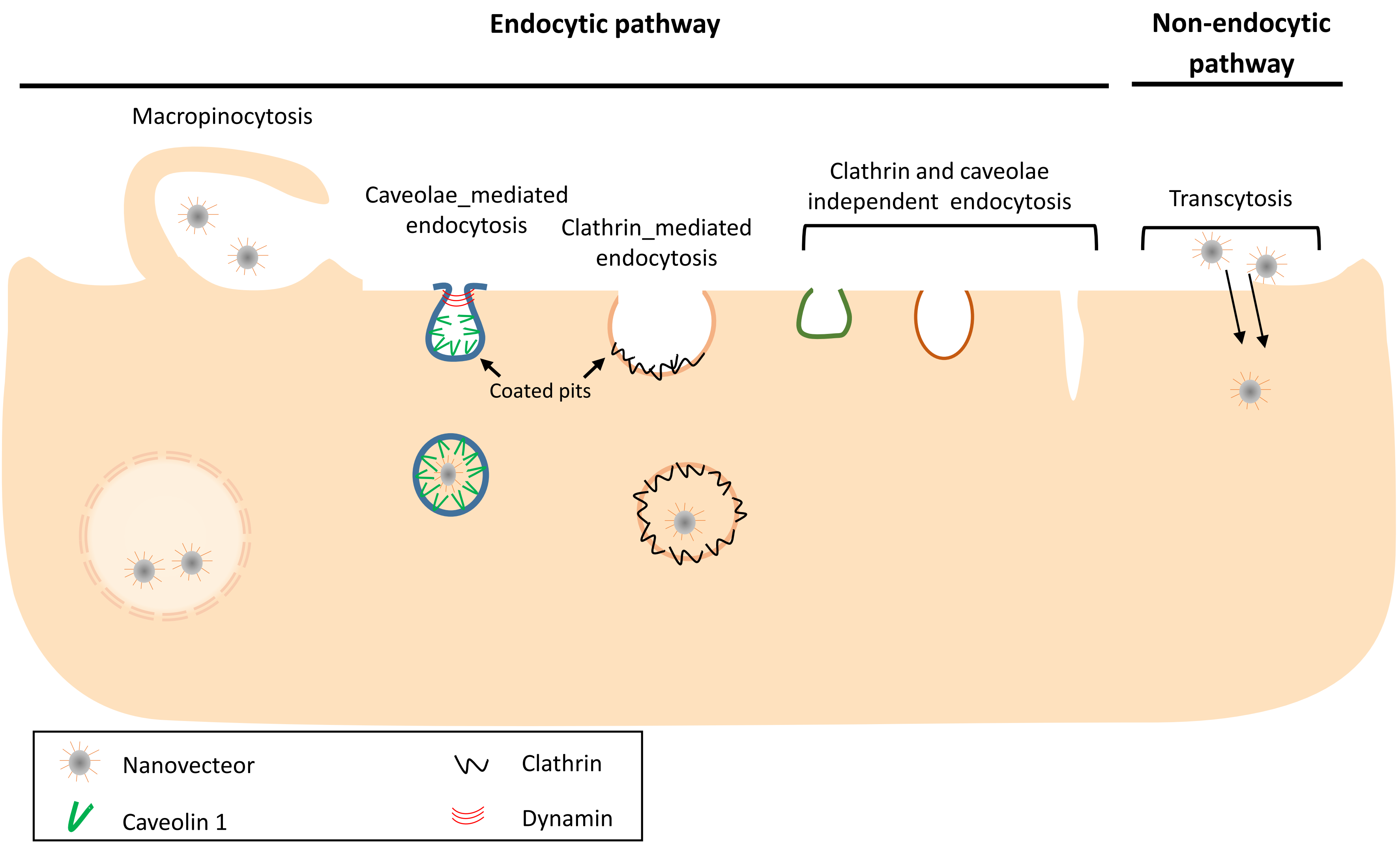
Figure 3. Schematic presentation showing extra- and intra-cellular trafficking of siRNA nanovectors after systemic administration. EPSN protect siRNA from enzymatic degradation in the blood. Thanks to their stealthiness, the extension of the circulation time and their characteristics, the accumulation in the tumor site is increased. The internalization of EPSN occurs by different routes, mostly via an endocytic pathway. When internalized by endocytosis, EPSN's components promote the endosomal escape of siRNA and avoid their lysosomal degradation to gain access to the cytosol where they use the mechanism of RNAi to down-regulate the expression of the target gene.

Figure 4. Schematic illustration of the different entry pathways of nanovectors. EPSN can be internalized via endocytic (macropinocytosis, caveolae-mediated endocytosis, clathrin-mediated endocytosis or clathrin and caveolae-independent endocytosis) or non-endocytic (transcytosis) pathway.

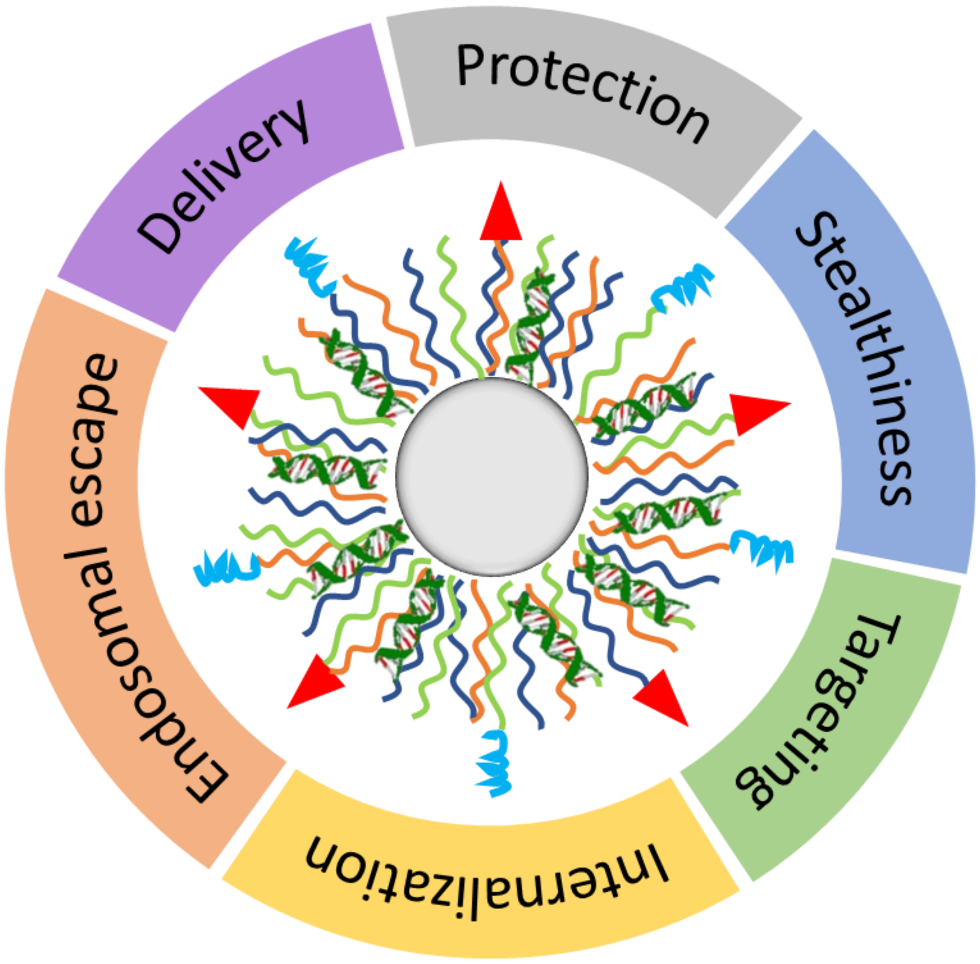

Enzymatic degradation
Early renal clearence
Low accumulation in tumor site
Repulsion
Cancer cell
Lysosome
Endosome
Blood vessel
Kidney
Tumor
Endosomal/lysosomal degradation


A
B
C
D
Polymers
siRNA
Peptide for active targeting
Cell-penetration peptide
Inorganic core


Protection from enzymatic degradation
Accumulation in tumor site
Internalization
Endosomal escape
Cancer cell
Lysosome
Endosome
nucleus
Blood vessel
Tumor
mRNA
Efficient down-regulation
Enzymes
siRNA nanovector
siRNA
mRNA translation



Endocytic pathway
Non-endocytic pathway
Macropinocytosis
Caveolae_mediated endocytosis
Clathrin_mediated endocytosis
Clathrin and caveolae independent endocytosis
Transcytosis
Coated pits
Nanovecteor
Clathrin
Caveolin 1
Dynamin


Protection
Stealthiness
Targeting
Internalization
Endosomal escape
Delivery