



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

S. Ben Djemaa, E. Munnier, I. Chourpa, E. Allard-Vannier, S. David*

Université de Tours, EA6295 Nanomédicaments et Nanosondes, 31 Avenue Monge, 37200 Tours, France

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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.

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 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 21–23 nucleotides, offers the best combination of specificity and potency as 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

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 nanovector

* Corresponding author.

E-mail address: stephanie.david@univ-tours.fr (S. David).

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hydrodynamic transfection method (Song et al., 2003). Today, some siRNA-based therapies for cancer treatment are in clinical trial phases (Nikam and Gore, 2018; Taberero 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, ONPATRO® (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 review 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 currently 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 (Fig. 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 the 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 system 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

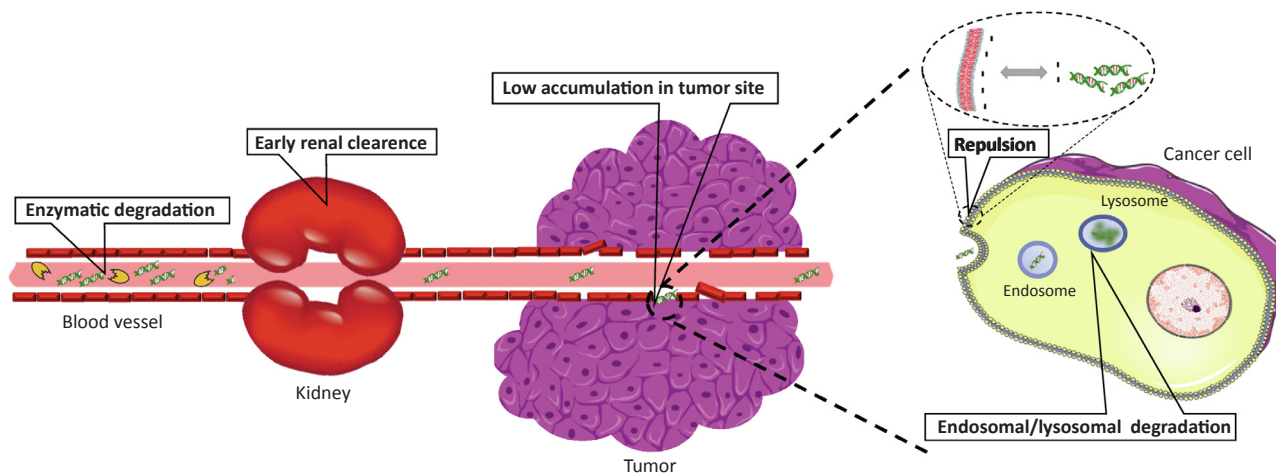


Fig. 1. Illustration of extra- and intra-cellular biological barriers for siRNA-based cancer therapy. Extracellular barriers: enzymatic degradation in the blood, early elimination by the kidney, low accumulation in the tumor site, and repulsion at the surface of the cell membrane. Intracellular barriers: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

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 (Fig. 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

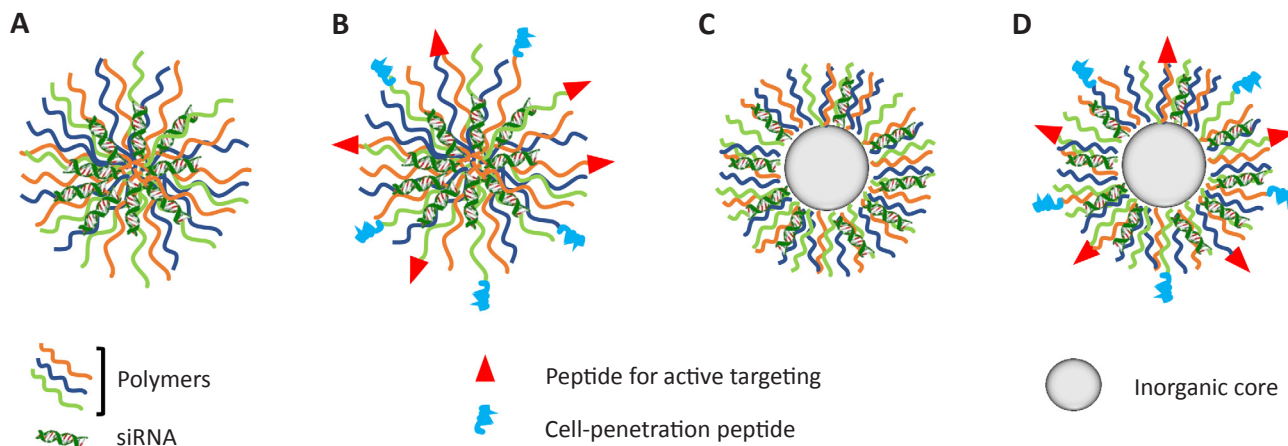


Fig. 2. Schematic presentation of electrostatically assembled polymer-based siRNA nanovectors (EPSN): (A) containing only polymers and siRNA, (B) decorated with peptides, (C) containing an inorganic core, and (D) containing an inorganic core and decorated with peptides.

(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 213 nm 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) (Pitella 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 (Fig. 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 4 h 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 and 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 at 37 °C, evaluated by a qualitative gel retardation assay (Liu et al., 2011). In addition to protect 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 their size remain 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

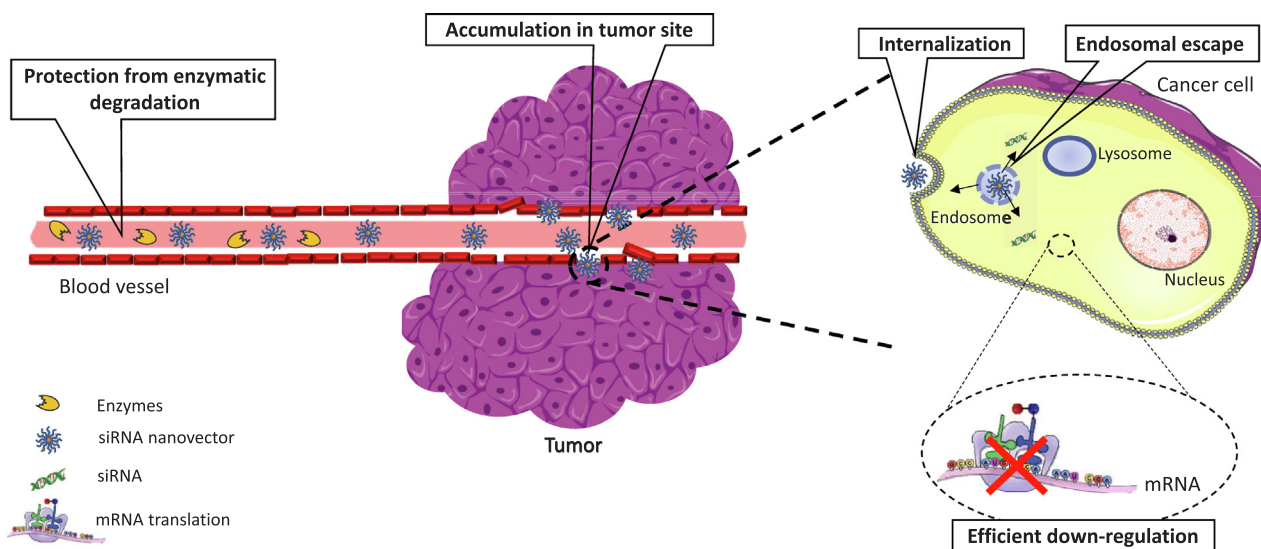


Fig. 3. Schematic presentation showing features of EPSN for efficient siRNA delivery after systemic administration. EPSN protect siRNA from enzymatic degradation in the blood. Thanks to their stealthiness, the extension of the circulation time and their characteristics, the accumulation in the tumor 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.

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 their accumulation due to the 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, siRNA can be associated to polymers to obtain complexes with adequate properties (50 nm < size < 250 nm and neutral charge) (Resnier et al., 2013). 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,b). 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 < 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-

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); Veisoh 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); Veisoh 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); Veisoh et al. (2011b)
Poly-lysine/poly-L-lysine	pLys/PLL	Cationic	10–70 KDa	Cavallieri et al. (2015); Jagannathan et al. (2014); Veisoh et al. (2011b)
Poly-alpha-glutaminate	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(DEF)	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 acid-co-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	Cavallieri et al. (2015); Pittella et al. (2011); Sun et al. (2016); Veisoh 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	GRQIKIWFQNRRMKWKK	No data	Muratovska and Eccles (2004)
gH625	gH625	Glycoprotein H of Herpes simplex virus type 1	Cell-penetrating peptide	HGLASTLIRWAHYNALIRAF	Not identified	Ben Djemaa et al. (2018)
Transportan	TP 10	Galanin and mastoparan	Cell-penetrating peptide	GWTLNSGYLLGKINIKALALAKKIL	No data	Pärnaste et al. (2017)
Chlorotoxin	CTX	Scorpion-derived peptide	Tumor-targeting peptide	MCMPCFITTDHQMARKDCCGGKGRGKCYGPOQLCR	affinity to a vast majority of brain tumors, prostate, skin and colorectal cancers	Mok et al. (2010); Veisoh et al. (2010)
Arginine-glycine-aspartate	RGD	Synthetic	Receptor-recognition motif	RGD	Tumor endothelial cells	Huang et al. (2015); Raggelle et al. (2015); Wang et al. (2009)
IL-4 receptor-binding peptide	IL4Rpep-1	Synthetic	Peptide	CRKRLDRNC	IL-4 receptor	Guruprasath et al. (2017)
RRRVVVVV	R3V6	Synthetic	Cell-penetrating peptide	RRRVVVVV	Not identified	Oh and Lee (2014)
Bombesin	BN	Skin of an European frog	Peptide	QLRGNQWAVGHLM	Gastrin-releasing peptide receptors	Wang et al. (2009)

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) (Fig. 4).

For EPSN constituted of siRNA complexed to polymers with or without inorganic core (Fig. 2A 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-lysine and PEG for the silencing of the anti-apoptotic gene, survivin, in prostate cancer cells. In this study, they showed that 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) (Fig. 2B 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 (Fig. 2B 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. Veisheh and coworkers have evaluated PEG-modified iron oxide nanoparticles coated with an oligo-arginine and loaded with

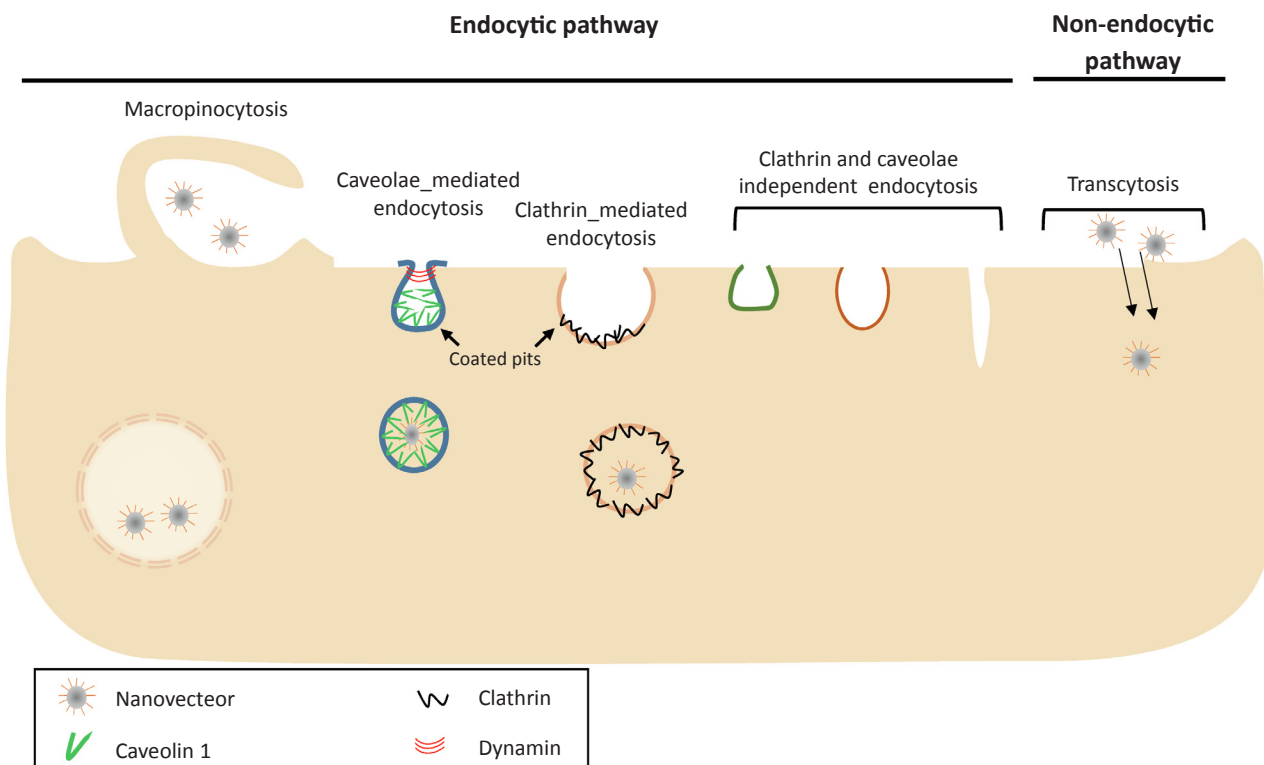


Fig. 4. Schematic illustration of the different entry pathways of nanovectors. EPSN can be internalized via endocytic (macropinocytosis, caveolae-mediated endocytosis, clathrin-mediated endocytosis or clathrin and caveolae-independent endocytosis) or non-endocytic (transcytosis) pathway.

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

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

Entry pathway	Nanovector	Techniques used for endosomal studies	Main result	Refs.
Receptor-mediated endocytosis	NP-siRNA-CTX	Fluorescence microscopy	Nanovectors are able to escape endosomes	Veisheh et al. (2010)
	HA- ^{PTX} /PSR _{siRNA}	Endosomal integrity assay: calcein	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.	Guruprasath et al. (2017)
		Confocal microscopy	After 24 h nanovectors are detected in late endosomes, lysosomes and cytosol	
	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	Pittrella et al. (2011)
	Mixed micelles	Confocal microscopy	Low colocalization with lysotracker	Miteva et al. (2015)
	NPEG – PLLs	Flow cytometry	Decrease of the colocalization of nanovectors with lysosomes after 24 h	Cavaliere 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: super-paramagnetic iron oxide nanoparticles; DG: 2-deoxyglucose; QDs: quantum dots; PEG: polyethylene glycol; CMCS: carboxymethyl chitosan; CaP: calcium phosphate; PLL: poly-L-lysine.

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 (Cavaliere et al., 2015)), apoptosis control (Bcl-2 family (Guruprasath et al., 2017)), cell cycle control, tumoral growth and angiogenesis (HIF 1 α (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 4 and 5 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. Veisheh 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 (Veisheh 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 (Veisheh 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% (Cavaliere et al., 2015; Miteva et al., 2015; Werfel et al., 2017; Xie et al., 2014). Veisheh 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% (Veisheh 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 (Veisheh et al., 2010) result in, respectively, 80% and 62% of GFP down-regulation. Similarly, the use of polylysine with modified PEG (Cavaliere 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

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 NIH3T3	Luciferase	100 nM	24	85	Werfel et al. (2017)
PEG-polyanion/siRNA/CaP hybrid nanoparticles	CaP, PEG, CCP, siRNA	MSC 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	4 T1	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	100 nM	48	79	Xie et al. (2014)
RGDp RI NP	PEG, chitosan, RGDp, siRNA	H1299	hTERT	100 nM	6	60	Corbet et al. (2016)
NP-pArg-siRNA	SPION, PEG, pArg, siRNA	SiHa	ASCT2	No data		70	
		C6	MCT1	No data	8	68	Veisch et al. (2011b)
		MCF7	GFP				
NP-siRNA-CTX	SPION, PEG, chitosan, PEI, chlorotoxin, siRNA	TC2	GFP	225 nM	2	62	Veisch et al. (2010)
NPEG - PLLs	NPEG-PLL, siRNA	C6	Survivine	31 nM	120	60	Cavaliere 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, lipotic acid- lysine- 9-poly-d-arginine (LA-Lys-9R), QDs, siRNA	Hep G2	GLUT1	50 nM	2	60	Zhu et al. (2015)
NP-pLys-siRNA	SPION, PEG, pLys, siRNA	C6	HIF-1 α	No data	8	39	Veisch et al. (2011b)
		MCF7	GFP				
NP-PEI-siRNA	SPION, PEG, PEI, siRNA	TC2	GFP	No data	8	32	Veisch et al. (2011b)
		C6					
		MCF7					
		TC2					

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); **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-lysine; **HA:** hyaluronic acid; **PTX:** Paclitaxel; **PSR:** Octyl modified polyethyleneimine containing disulfide linkages; **DG:** 2-deoxyglucose; **QDs:** quantum dots; **pLys:** polylysine.

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)	4 T1-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 RI 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-9R), QDs, siRNA	HepG2 cells Kunming mice	HIF_1α	3 mg/kg	8 (1 day/2)	Intravenously	No data	Zhu et al. (2015)

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.

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 (Tables 4 and 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 (Tables 4 and 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 150 nM (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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Declaration of Competing Interest

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

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