

1 **Versatile electrostatically assembled polymeric siRNA nanovectors:** 2 **can they overcome the limits of siRNA tumor delivery?**

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8 **Keywords**

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

11 **Abbreviations**

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

22 **Abstract**

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

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59

60 **1. Introduction**

61 Since the 1990s and the discovery of post-transcriptional gene extinction in plants (Ratcliff et al.,
62 1997), the mechanism of RNA interference (RNAi) has gained scientists' interest worldwide. This
63 discovery has provided hope for the treatment of many severe diseases like cancers, autoimmune
64 diseases, dominant genetic disorders and viral infections (Ferrari et al., 2012). RNAi is a natural
65 phenomenon of sequence-specific gene silencing mediated by short sequences of non-coding
66 endogenous RNA such as small hairpin RNA (shRNA), micro RNA (miRNA) and small interfering RNA
67 (siRNA), considered as regulation systems of gene expression and RNA-based gene-silencing
68 molecules (Carthew, Richard W. and Sontheimer et al., 2009). The use of RNAi is linked to the
69 transfer of genetic material into damaged cells, in order to ensure a targeted molecular intervention
70 and achieve a higher level of specific action than conventional cytotoxic chemotherapy (Jabir et al.,

71 2012). Basically, thanks to the potency and the selectivity for the silencing of specific genes, RNAi-
72 based therapy could treat any human disease caused by the over-expression of one or few genes
73 (Aagaard and Rossi, 2007). Over the past few years, different approaches have been developed
74 based on this strategy to inhibit the expression of certain genes, called oncogenes, coding for over-
75 expressed proteins that are implicated in tumor growth (Haussecker, 2014). Among these small
76 nucleic acids, it is generally accepted that siRNA sequences, of 21 to 23 nucleotides, offers the best
77 combination of specificity and potency as a therapeutics and are the most used in the development
78 of anticancer treatments (Ferrari et al., 2012; Resnier et al., 2013)

79 Following the demonstration of RNAi in mammalian cells in 2001 (Elbashir et al., 2001), several
80 studies have quickly concentrated on specific gene silencing of siRNA to exploit this powerful
81 mechanism to interfere with cancer-causing or cancer-promoting genes to develop a new class of
82 drugs. In 2003, Song et al. presented the first *in vivo* evidence of RNAi-based therapeutic efficacy to
83 protect mice from liver failure and fibrosis. By using siRNA duplexes targeting gene Fas, they
84 demonstrated a specific decrease of mRNA level and protein expression of Fas in mice hepatocytes,
85 after intravenous injections using a modified hydrodynamic transfection method (Song et al., 2003).
86 Today, some siRNA-based therapies for cancer treatment are in clinical trial phases (Nikam and Gore,
87 2018; Tabernero et al., 2013; Tatiparti et al., 2017). For example, Silenseed Ltd performed the phase I
88 clinical trial of its siRNA-based treatment (siG12D-LODER) against pancreatic cancer. SiG12D-LODER
89 target the oncogene KRAS that is implicated in cancer growth. This trial was completed in 2014 and
90 showed high safety and tolerability profiles of this treatment in patients. This therapy is currently in
91 Phase II trial which aims to evaluate the efficacy of the combination of siG12D-LODER with standard
92 chemotherapy treatment (Gemcitabine + nab-paclitaxel) by measuring progression-free survival in
93 patients (Kaczmarek et al., 2017). The first siRNA-based therapy, ONPATPRO® (patisiran), was
94 approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in
95 August 2018 for the treatment of polyneuropathy of hereditary transthyretin-mediated amyloidosis

96 in adult patients (Al Shaer et al., 2019; Rizk and Tüzmen, 2017). This approval presents a great
97 achievement in nanomedicine discovery and development and provides hope for the progress
98 toward an anti-cancer application.

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

107 **2. siRNA delivery: challenges and nanovectorization**

108 **2.1. Extra- and intracellular barriers for naked siRNA**

109 The limitations of naked siRNA are due to their properties (charge, hydrophilicity, size, sensitivity to
110 degradation ...) which represent hurdles in each step of the trafficking of siRNA, extra- and
111 intracellularly (Figure 1). For example, in the blood or in biological environment, presence of enzymes
112 such as ribonucleases affects the siRNA stability and involves their rapid degradation (Gavrilov and
113 Saltzman, 2012). Even if siRNA escape enzymatic degradation in blood, their small size favors their
114 rapid elimination by renal clearance. Therefore, the accumulation of siRNA in target site is a big
115 challenge. Furthermore, the characteristics of the tumor tissue, such as i) the heterogeneous blood
116 flow distribution and poor perfusion of inner region of solid tumor, ii) the dense intercellular matrix
117 in this region, and iii) high hypoxia, acidity and interstitial fluid pressure, due to dysfunctional tumor
118 lymphatics (Forster et al., 2017; Gillies et al., 1999; Heldin et al., 2004), restrict the uniform delivery
119 of nanovectors to the tumors in sufficient quantities (Jain and Stylianopoulos, 2010). Once in the

120 tumor site, siRNA must reach their target cells that express or overexpress the gene(s) of interest.
121 Nevertheless, naked siRNA do not have the ability to distinguish target cells and they can act in the
122 same way on normal cells and defective cells including unwanted off-target effects (Wang et al.,
123 2017). Moreover, the negative charge of siRNA phosphate groups and their hydrophilicity limit their
124 ability to cross cell membranes, because of the electrostatic repulsions between siRNA and the cell
125 surface, negatively charged as well, and the impermeability of the lipid bilayer to hydrophilic
126 molecules (Dominska and Dykxhoorn, 2010; Reischl and Zimmer, 2009; Videira et al., 2014). The
127 small amount of siRNA that overcomes previously mentioned challenges and is internalized into cells
128 must escape endosomal/lysosomal degradation in order to reach cytosol where its targets are
129 present (Gavrilov and Saltzman, 2012). In addition to these limitations, the immunogenicity of siRNA
130 represents another concern associated with *in vivo* administration. In fact, Reynolds *et al.* reported
131 that siRNA can activate the innate immune reaction by inducing the expression of associated genes
132 such as interferons or interferon-inducible genes. They demonstrated that this activation is cell type-
133 and siRNA length-dependent (Reynolds et al., 2006). Other studies showed that certain siRNA can be
134 recognized by some Toll-like receptors (TLR) such as TLR3, 7 and 8. This recognition can trigger
135 interferon pathway responses (Behlke, 2006). The activation of this pathway results in the induction
136 of the apoptosis and the cell death (from 20% to 60% of cell death) (Reynolds et al., 2006). The
137 consequence of the association of previously presented limitations of the use of naked siRNA is an
138 unsatisfactory effect *in vitro* as well as *in vivo*. It is, therefore, necessary to develop delivery systems
139 with suitable properties to overcome all these challenges.

140 **2.2. Nanovectorization for siRNA delivery**

141 Because of the challenges mentioned above, many approaches were adopted to develop various
142 galenic forms of siRNA-based medicine, in order to exploit the powerful effect of siRNA in anticancer
143 therapies. One promising approach is the loading of siRNA in a nanovector. This strategy, called
144 siRNA nanovectorization, consists in associating siRNA to suitable materials to obtain a nano-sized

145 vector able to effectively convey siRNA toward their target (Ferrari et al., 2012; Resnier et al., 2013).
146 Nanovectors are developed to carry and deliver drugs, oligonucleotides, peptides or other desired
147 cargos to target tissues. Various nanosystems have been used for siRNA delivery in biomedical
148 applications. At the present time, a relatively extensive arsenal of nanovectors has been proposed to
149 administer siRNA without interfering with their silencing efficiency (Ozcan et al., 2015). In literature
150 several types of nanovectors are described, including organic (lipid-based, polymer-based, peptide-
151 based) (Resnier et al., 2013) and inorganic ones (based on the use of iron oxide, gold, quantum dots,
152 ...) (Conde et al., 2014). These nanovectors can be associated with siRNA using various methods: 1)
153 conjugation, which needs chemical intervention and consists in covalently attaching siRNA to the
154 nanovector components (Ding et al., 2014, 2012; Muratovska and Eccles, 2004), 2) encapsulation
155 that is based on the loading of siRNA into a protective shell (liposomes or micelles, for instance)
156 (Chen et al., 2012; David et al., 2012; Mokhtarieh et al., 2018) and 3) electrostatic bonds which aim
157 to complex negatively charged siRNA with positively charged nanovector components (Bruniaux et
158 al., 2017; Guruprasath et al., 2017; Liu et al., 2011).

159 **3. Electrostatically assembled polymeric siRNA nanovectors (EPSN)**

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

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

186 Despite the ease and the speed of the preparation of electrostatically assembled nanovectors, their
187 development needs serious work on the optimization of the formulation. Indeed, a critical point is
188 the stability of these complexes in biological environment. As the electrostatic association between
189 siRNA and cationic components is low, these complexes may disassemble too early if the formulation
190 is not optimized (Creusat et al., 2010; Creusat and Zuber, 2008). Therefore, to successfully complex
191 siRNA with polymers, there are some parameters to consider such as the components ratio and
192 concentration (Richards Grayson et al., 2006). One can distinguish two types of ratio described in

193 literature: i) charge ratio which represents the molar ratio between the number of positive charges
194 of polymers (for instance, those of amino groups) and that of negative charges of siRNA phosphate
195 groups (N/P ratio) (Guruprasath et al., 2017; Werfel et al., 2017), and ii) mass ratio which represents
196 the ratio between the mass of polymer and that of siRNA (Corbet et al., 2016; Veiseh et al., 2010).
197 These ratios are usually optimized at the beginning of each siRNA nanovector development to
198 determine the best formulation. The complexation efficiency is often evaluated by gel retardation
199 assay using ethidium bromide, as nucleic acid intercalant (Liu et al., 2011; Veiseh et al., 2011b, 2010).
200 In fact, with this technique, free siRNA appear as fluorescent bands, while no fluorescence is
201 detected if they are complexed and not accessible to ethidium bromide.

202 siRNA can also be complexed with polymers surrounding an inorganic core or support (Ben Djemaa
203 et al., 2018; Guruprasath et al., 2017; Pittella et al., 2011). In this case another ratio appears which
204 defines the quantity of the inorganic part used in the nanovector. The presence of the inorganic core
205 can be advantageous for the formulation. In fact, it has been demonstrated that the use of the
206 inorganic core (based on superparamagnetic iron oxide nanoparticles (SPION)) plays an important
207 role in the stability and the control of the size of the final nanovector. Addition of an inorganic core
208 to the formulation decreased the size and the polydispersity index of the complexes from 213nm to
209 175 nm and from 0.43 to 0.34, respectively (Ben Djemaa et al., 2018). Moreover, Xie *et al.* developed
210 hybrid nanoparticles based on calcium phosphate core for the electrostatic loading and delivery of
211 siRNA. These nanoparticles exhibited efficient siRNA loading and enhanced colloidal and serum
212 stability (Xie et al., 2014). Apart from their role in the formulation and the transport of siRNA, some
213 inorganic cores such as quantum dots (Derfus et al., 2007; Pan et al., 2010), gold nanoparticles
214 (Jaganathan et al., 2014; Rosi et al., 2006; Song et al., 2010) or magnetic nanoparticles (Lu et al.,
215 2007; Sun et al., 2008), can also be used as diagnostic tools. They allow the monitoring and the study
216 of the distribution of inorganic nanovectors [45,46] using fluorescence, fluorescent energy transfer

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

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

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

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

241 **3.1. Design rationale of EPSN to overcome extracellular barriers**

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

246 **3.1.1. Protection from enzymatic degradation and premature clearance**

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

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

264 Another example of cationic polymers classically used and studied for gene delivery/therapy is
265 Polyethylenimine (PEI). PEI is a synthetic macromolecule consisting of a repeating amine and ethyl
266 unit, with a high cationic charge density able to condense spontaneously, via electrostatic
267 interaction, anionically charged siRNA and increase their stability in biological medium (Boussif et al.,
268 1995). In their study, Liu et colleagues have successfully complexed siRNA with Alkyl-PEI. This
269 complexation results in a high siRNA protection from enzymatic degradation in the presence of 50%
270 of serum and at 37°C, evaluated by a qualitative gel retardation assay (Liu et al., 2011). In addition to
271 protecting the siRNA from the degradation and the early elimination, these polymers are able to
272 condense nucleic acid and increase the size of the complex, compared to the size of naked siRNA, to
273 avoid the clearance of siRNA, while getting a relatively small size, suitable for gene delivery (Arnold et
274 al., 2017; Parmar et al., 2018; Videira et al., 2014).

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

287 **3.1.2. Increase of the immune stealthiness of siRNA**

288 One of the major bottlenecks of the use of siRNA is their immunogenicity and their negative charge.
289 These limitations underline the importance of an improved strategy for the delivery of siRNA. The
290 complexation of siRNA with polymers could be an approach to overcome this challenge. Takeshita
291 and coworkers used atelocollagen for the intravenous delivery of siRNA in a bone tumor metastasis
292 model in mice. After the injection of a control naked siRNA or atelocollagen-siRNA complex, they
293 evaluated the stimulation of the innate immune responses and they showed that the association of
294 siRNA with this polymer did not result in an increase in the level of interferon (Takeshita et al., 2005).
295 Moreover, the association of siRNA to polymers could neutralize their negative charge. As an
296 example, the electrostatic assembly of siRNA with Alkyl-PEI or with a complex of polymers and
297 peptide result in neutral zeta potential of the nanovectors around -2.6 or -0.01 mV, respectively (Liu
298 et al., 2011; Veiseh et al., 2011b). However, the use of several cationic polymers or highly positively
299 charged polymers results in unwanted high density of positive charges. In fact, a high positive surface
300 charge induces the interaction with negatively charged plasmatic molecules and the formation of
301 large aggregates that can be recognized by the innate immune system and promotes their
302 elimination (Resnier et al., 2013). In EPSN containing an inorganic core, the association of neutral
303 polymers such as PEG or polyvinylpyrrolidone (Pan et al., 2018) is often chosen to mask charges and
304 to increase the stealthiness of siRNA nanovectors (Arnold et al., 2017). Neutral polymers are usually
305 attached by covalent interaction to the inorganic core of the nanovector (Veiseh et al., 2011b), to
306 cationic polymers (Xie et al., 2014) or to both of those (Veiseh et al., 2010). For example, in one
307 study, the use of polylysine to develop a siRNA nanovector for the targeting of breast tumor-initiating
308 cells yielded in positive zeta potential of 19 mV. In contrary, the addition of PEG to polylysine in
309 another siRNA nanovector resulted in a surface charge of 0.5 mV. By masking the surface charge of
310 nanovectors, PEG is able to avoid siRNA nanovectors' binding to plasma proteins, prolong their
311 systemic circulation time, prevent their recognition by the immune system and promote an

312 enhanced permeability and retention (EPR) effect in different types of tumors (Jabir et al., 2012;
313 Owens III and Peppas, 2006). Sun *et al.* showed that PEGylation of their polymeric siRNA nanovector
314 using PEG_{5k} or PEG_{6k} prolonged the circulation time in the blood 4-fold compared to free siRNA, by
315 preventing protein adsorption on the surface (Sun et al., 2015).

316 **3.1.3. Targeting of cancer cells**

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

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

337 Peptides, or polyamines, are short chains containing less than 50 amino acids monomers linked by
338 amide bonds and are structurally similar to proteins. Peptides can be found naturally or synthetically
339 and have the potential for the stabilization and biofunctionalization of nanoparticles (Conde et al.,
340 2014; Zhang et al., 2016). Peptides can be associated with siRNA nanovectors by electrostatic or
341 covalent bonds (Corbet et al., 2016; Jiang et al., 2012; Muratovska and Eccles, 2004; Wang et al.,
342 2009). Table 2 presents some of the most used peptides in the functionalization of siRNA
343 nanovectors. Some peptides can be selectively addressed to membrane molecules on the surface of
344 specific cells (Conde et al., 2014; Schmohl et al., 2017). Thus, the use of these peptides in siRNA
345 nanovectors could guide and improve the interactions with cell surfaces. In order to treat cancer,
346 Guruprasath and colleagues presented an example of the functionalization of siRNA nanovectors
347 with peptide for active targeting. In this study, they demonstrated a specific interaction of their
348 siRNA nanovector functionalized with Interleukin-4 receptor (IL-4R)-binding peptide 1 (IL4RPep-1)
349 with the IL-4R up-regulated on cancer cells. Furthermore, they showed an efficient accumulation in
350 tumor, 3-fold more than with nanovectors without peptide (Guruprasath et al., 2017).

351 To enhance the targeting and the penetration in the tumor site, a rational design of nanovectors that
352 considers tumors characteristics and the properties of their microenvironment, mentioned in section
353 2.1., is needed. As solid tumors exhibit low interstitial pH, many pH-sensitive nanovectors were
354 developed to deliver siRNA to tumors using pH-sensitive peptides for instance (Mok et al., 2010; Zhu
355 et al., 2015). In addition, the formulation of hypoxia-sensitive nanovectors using, for example,
356 hypoxia-responsive polymers or hypoxia-targeted polymers can be used to benefit from the hypoxia
357 in tumor site (Kang et al., 2016; Perche et al., 2016). Perche and colleagues synthesized hypoxia-
358 sensitive polymers to develop a nanovector for the delivery of siRNA in tumors. They showed that
359 these polymers respond to the hypoxia-stimulation by detaching PEG from the complexes to enhance
360 the accessibility and the targeting of tumor cells (Perche et al., 2014). In tumors, the deep
361 penetration of nanovectors can also be achieved by the application of an external magnetic field

362 thanks to the presence of iron magnetic nanoparticles in the formulation of the nanovector (Scherer
363 et al., 2002).

364 **3.2. Design rationale of EPSN to overcome intracellular barriers**

365 **3.2.1. Cellular uptake of EPSN**

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

371 **3.2.1.1. Internalization by endocytosis**

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

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

396 The functionalization of the surface of EPSN with peptides can help to enhance passage through the
397 membrane mediated by active endocytosis (Azevedo *et al.*, 2018) (Figure 2 B and D). Some peptides
398 used for the functionalization of siRNA nanovectors are able to recognize specific molecules on the
399 cell membrane such as receptors. Upon binding to these molecules, the entry of associated siRNA
400 nanovector occurs by receptor-mediated endocytosis. This internalization pathway is largely used for
401 active targeted siRNA delivery. In this process, receptors are considered as mediators between cells
402 and extracellular molecules/particles, they play a crucial role in cellular internalization by ensuring
403 high specific interaction. Although numerous mechanisms of ligand-receptor internalization exist, all
404 occur by ligand-stimulated manner. Briefly, the binding of ligand, held on nanovectors surface, to the
405 extracellular domain elicits the receptor phosphorylation. Following this step, the phosphorylated
406 receptor-ligand binary complex or only the phosphorylated receptor is internalized (Allen, 2002). In
407 the case of nanovectors, it is requested to be receptor-ligand internalization. Depending on ligand
408 nature and cell type, intracellular processing of ligand can differ. Although internalized ligands
409 (likewise peptide functionalized nanovectors) commonly end into endosomal compartment, receptor

410 is recycled back to the cell membrane (Lodish et al., 2000; Prokop and Davidson, 2008). Indeed, in
411 endosomes, the recruitment of vacuolar ATPase pump causes vesicles acidification by the entry of H⁺
412 ions. The acidic pH induces a conformational change of receptors, often resulting in a ligand-receptor
413 dissociation (Bareford and Swaan, 2007). This mechanism can be considered as the best entry route
414 for a high targeting specificity and an efficient cellular uptake of nanovectors. As an example for this
415 entry pathway, Guruprasath and coworkers functionalized their siRNA nanovector by IL-4 receptor-
416 binding peptide (IL4RPep-1) to target IL-4R for the delivery of anti-Bcl-xL siRNA. Results showed a
417 high accumulation of the siRNA nanovector in the tumor and a specific internalization by IL-4
418 receptor-mediated endocytosis (Guruprasath et al., 2017).

419 **3.2.1.2. Internalization mediated by transcytosis**

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

424 Transcytosis is a mechanism allowing to cross the cell membrane in an energy independent way. It
425 depends on the size, the charge and the nature of nanovector surface components and on the
426 nanovector concentration (Tuma and Hubbard, 2003). Peptide-functionalized nanovectors, in
427 particular those conjugated to cell-penetrating peptides (CPP), have various internalization
428 mechanisms. CPP are short peptide sequences of about thirty amino acids positively charged and are
429 known for their ability to cross the lipid membrane by translocation mediated with their hydrophobic
430 sequence and directly enter the cytosol (Rothbard et al., 2004). Briefly, the amphipathic character
431 and the easy change of CPP structure from α -helices to β -sheets provide this peptide a high degree of
432 conformational flexibility. This property has a key role in CPP translocation capacity. CPP – mediated
433 transcytosis is induced by CPP hydrophobic extremity, so-called membrane perturbing/interacting
434 domain. This extremity initiates lipid destabilization of cell membranes which permits the fusion with

435 lipid bilayer in order to gain the cytoplasmic compartment (Galdiero et al., 2015). These short
436 amphipathic peptides are emerging as attractive gene delivery tools and they can be associated with
437 other molecules of different nature such as polymers (Wang et al., 2014). One example of the
438 application of such a short peptide was published by Oh *et al.* who used the CPP R3V6 associated by
439 electrostatic manner to deliver siRNA against sphingosine-1-phosphate lyase (S1PLyase) and
440 recombinant high mobility group box-1 box A peptide (HMGB1A) into LA-4 lung epithelial cells in
441 animal model. The presence of R3V6 increases the cell entry of the nanovector (Oh and Lee, 2014).
442 Despite the absence of specific tumor recognition, this study showed that the use of CPP improves
443 siRNA delivery, indicating the participation of the EPR effect. Once nanovectors are accumulated, the
444 CPP intervenes to enhance the deep penetration into tumor cells. Veiseh and coworkers have
445 evaluated PEG-modified iron oxide nanoparticles coated with an oligo-arginine and loaded with
446 siRNA (size about 50 nm) for their cellular entry pathway in three types of cancer cells. Results
447 showed an enhanced internalization of this siRNA nanovectors by transcytosis without the formation
448 of endocytic vesicles (Veiseh et al., 2011b).

449 **3.2.2. Endosomal escape**

450 Due to their endosomal buffering ability, cationic polymers can facilitate the endosomal escape of
451 siRNA. Most EPSN are internalized by endocytosis, more precisely pinocytosis (Corbet et al., 2016; Xie
452 et al., 2014; Yin et al., 2016). Briefly, after immobilization on the cell surface, nanovectors are
453 encompassed in vesicles derived from local invagination of the cell membrane. After vesicles
454 formation, nanovectors are attracted into cell inside newly formed endosomes (Wang et al., 2010). In
455 this stage, the challenge of siRNA nanovectors is to escape endosomes before their fusion with
456 lysosomes to avoid degradation and to pass into the cytosol. At this level, cationic polymers could be
457 good candidates for this challenge. In 1997, Behr and others introduced the concept of the proton
458 sponge and hypothesized that polymers such as PEI, polylysine and polyarginine could buffer the
459 acidity of endosomes and induce their rupture (Behr, 1997). Afterward, this concept was more

460 studied and developed. To summarize, endosomes acidification causes two complementary and
461 simultaneous effects. The first is the so-called “proton sponge effect” which consists of a massive
462 entry of water following a high concentration of hydrogen chloride (HCl) caused by the stimulation of
463 the flow of chloride ions after the increase of the H⁺ ions density in endosomes. The second is the
464 consequence of the acidification of the endosomes and is called the umbrella effect that occurs by
465 the capture of positive charges by cationic components of nanovectors, inducing thus an increase in
466 the volume occupied by these molecules caused by the repulsions between groups of the same
467 charge. These two phenomena combined allow the lysis of the endosomes (Nguyen and Szoka, 2012)
468 and promote the passage of nanovectors and/ or siRNA into the cytosol. Recently, the proton sponge
469 hypothesis was discussed on the part of the lysis of the endosomal membrane. Several studies
470 showed that this complete rupture is highly unlikely and that in the presence of cationic polymers,
471 the endosomal escape is promoted by the interaction of polymers’ amino groups and the inner side
472 of the membrane. This interaction causes a local membrane destabilization which leads a transient
473 formation of “nanoscale holes” which could explain the endosomal escape (Jonker et al., 2017;
474 Rehman et al., 2013; Schubert et al., 2018; Trützscher et al., 2018). In their study, Xie *et al.* used an
475 inorganic core of calcium phosphate and a polymer coating (PEG and modified chitosan) to
476 nanovectorize siRNA. They demonstrated that the nanovector was internalized mainly by
477 macropinocytosis with the contribution of clathrin- and caveolae-mediated endocytosis. Using
478 fluorescently labeled siRNA loaded in their nanovector, endosomal-lysosomal tracker and confocal
479 laser scanning microscopy, they observed colocalization between the fluorophore associated to
480 siRNA and that of the tracker after 3 h of nanovector incubation with cells. However, after 6 h the
481 colocalization of these fluorescent signals was decreased, and fluorescent siRNA was detected in the
482 cytoplasm. Authors explained this observation by the dissociation of the calcium phosphate core
483 from polymers due to the protonation of amino groups of PEG-chitosan in the acidic environment.
484 This process leads to the swelling of endosomes and then the release of siRNA into the cytoplasm

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

487 **3.3. Evaluation of protein down-regulation efficiency**

488 The efficiency of siRNA nanovectors is evaluated by the cellular and/or the molecular responses of
489 treated cells or tissues and it depends on the used siRNA. The evaluation of the molecular response
490 can reflect the efficiency of the nanovector even if there is no cellular effect of the used siRNA.
491 Molecular responses are the inhibition of the targeted mRNA expression and consequently a
492 decrease in the expression of the associated protein. In the development phase of a nanovector,
493 model siRNA (or reporter siRNA) targeting GFP or luciferase are widely used because they are
494 convenient, relatively inexpensive, and gives quantitative and rapid measurements. These siRNA are
495 commonly used as a tool to study gene expression at the transcriptional level and they give a
496 molecular response due to the inhibition of the GFP or the luciferase protein and the extinction of
497 their signals, easily detected by flow cytometry (for GFP) or luminescence (for luciferase) analysis. In
498 the validation phase, the cellular response is usually an induction of cell death and it is detected by
499 cytotoxicity (WST-1, MTT, LDH, ...) or apoptosis assays (Annexin V- FITC / PI assay, DNA laddering, ...).
500 The used siRNA usually target mRNA of genes implicated in different functions needed for tumor
501 process such as cell survival (survivin (Cavalieri et al., 2015)), apoptosis control (Bcl-2 family
502 (Guruprasath et al., 2017)), cell cycle control, tumoral growth and angiogenesis (HIF 1 α (Zhu et al.,
503 2015)), tumor cells migration, metastasis (VEGF (Chen et al., 2014)), etc. The efficiency of a
504 nanovector depend on a) the nanovector design and composition, b) the chosen cellular model and
505 the corresponding target protein and c) the chosen therapeutic scheme. Tables 5 and 6 give an
506 overview of some examples of existing versatile polymeric nanovectors which efficiently down-
507 regulate protein expression.

508 **3.3.1. Influence of the nanovector design and composition**

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

523 Several studies showed that the chosen polymers could affect the stability, the trafficking and,
524 therefore, the efficiency of the siRNA nanovector. For example, siRNA nanovectors containing PEG as
525 a neutral polymer to increase their colloidal stability and their stealthiness show, generally, a
526 transfection efficiency higher than 60% (Cavaliere et al., 2015; Miteva et al., 2015; Werfel et al., 2017;
527 Xie et al., 2014). Veiseh and coworkers evaluated PEG-modified iron oxide nanoparticles coated with
528 either polyarginine, polylysine or PEI for their ability in promoting gene knockdown by siRNA
529 delivery. They demonstrated that the transfection efficiency depended on the used cationic polymer.
530 In fact, it was inferior to 40% by using polylysine or PEI as the only cationic polymer in the
531 formulation. However, the replacement of these two polymers by polyarginine increases the
532 efficiency of the nanovector to 68% (Veiseh et al., 2011b). In other studies, the use of PEI and

533 polylysine in siRNA nanovectors with more complex structures showed a high down-regulation
534 efficiency. For example, the use of PEI with chitosan, PEG and a small peptide (Ragelle et al., 2015) or
535 with SPIONs, chitosan, PEG and chlorotoxin peptide (Veiseh et al., 2010) result in, respectively, 80%
536 and 62% of GFP down-regulation. Similarly, the use of polylysine with modified PEG (Cavalieri et al.,
537 2015) or with PEG, polyarginine and quantum dots (Zhu et al., 2015) results in 60% of transfection
538 efficiency.

539 **3.3.2. Influence of the cellular model and the corresponding target protein**

540 Model cells used to evaluate the down-regulation efficiency of siRNA nanovectors are always chosen
541 to be representative of the targeted cancer type (Table 4). The cellular responses towards gene
542 therapies depend on the cell type. In this context, Veiseh *et al.* evaluated *in vitro* the transfection
543 efficiency of EPSN based on the use of PEGylated superparamagnetic iron oxide nanoparticles
544 (SPION) polyarginine in cell lines expressing GFP representative of glioma, breast cancer and colon
545 adenocarcinoma: C6, MCF7, and TC2 respectively. These nanovectors appear to be significantly more
546 efficient to down-regulate the expression of the GFP in MCF7 cells (68.2 %), followed by C6 cells
547 (52.9%) and TC2 cells (24%) (Veiseh et al., 2011b). Similarly, Werfel and coworkers showed that the
548 transfection efficiency of a siRNA nanovector formulated using DMAEMA, BMA and PEG as polymers
549 and siRNA anti luciferase at a concentration of 100 nM varies in three cell lines: MDA-MB-231,
550 NIH3T3 and mesenchymal stem cell (MSC), but it was higher than 80 % in all the cell lines (Werfel et
551 al., 2017).

552 As we mentioned above, generally, in the development stage of siRNA nanovectors it is easier to use
553 a model gene, but then it is necessary to evaluate the silencing potential of the siRNA nanovector on
554 a target gene, usually related to the tumor process (Table 4 and Table 5). However, the modification
555 of the target protein leads, sometimes, to a variable down-regulation efficiency dependent on the
556 protein. For example, Xie *et al.* evaluated the transfection efficiency of a siRNA nanovector prepared
557 with an inorganic core of calcium phosphate nanoparticles and a coating of PEG grafted

558 carboxymethyl chitosan on HepG2 model cells expressing luciferase at a siRNA concentration of 100
559 nM. The incubation of this nanovector prepared with siRNA anti-luciferase leads to 79% of silencing
560 efficacy. However, the evaluation of the therapeutic potential of siRNA delivery targeting hTERT gene
561 results in only 60% and almost 50 % of inhibition in the targeted mRNA and protein level (Xie et al.,
562 2014). This study showed a loss of down-regulation efficiency of at least 20% between the model
563 gene and the gene of interest.

564 **3.3.3. Influence of the therapeutic scheme**

565 **3.3.3.1. Dose of siRNA**

566 The dose or the concentration of siRNA is one of the important parameters to consider for successful
567 gene transfection and satisfactory gene silencing (Table 4 and Table 5). The determination of the
568 adequate siRNA quantity requires an optimization step. Ragelle and colleagues performed a
569 transfection of cells with an EPSN at different siRNA concentrations (from 12.5 nM to 200 nM). They
570 showed that at low concentration (12.5 – 50 nM) the gene silencing of GFP was lower than 40% and
571 it increased significantly up to 150 nM of siRNA to achieve almost 90%. However, no significant
572 increase in the silencing efficiency was observed at concentrations above 150nM (Ragelle et al.,
573 2015). Moreover, the used concentration of siRNA depends on the used nanovector. In fact, by using
574 different EPSN the same down-regulation efficiency can be achieved, but with different siRNA
575 concentrations. For example, to obtain 80% of silencing of luciferase in breast cancer cells, Liu and
576 colleagues used 6 pmol of siRNA loaded in a nanovector based on iron oxide nanoparticles and alkyl-
577 PEI (Liu et al., 2011). However, for the same luciferase silencing efficiency (80%), Miteva *et al.* used a
578 siRNA nanovector prepared with two polymer blocks (PEG-b-pDPB et pD-b-pDPB) at a siRNA
579 concentration of 100 nM (Miteva et al., 2015), much higher than the previous study. Likewise, the
580 intravenous administration of vectorized siRNA (with DMAEMA, BMA and PEG) in a xenograft mouse
581 cancer model at a concentration of 1 mg/kg resulted in 59 % of efficiency (Werfel et al., 2017). Yet,
582 Corbet *et al.* obtained almost the same efficiency (60%) by injecting by the same route a siRNA

583 nanovector prepared with two polymers, PEG and chitosan, and functionalized with the peptide RGD
584 in a xenograft mouse cancer model at a dose twice as high (2 mg/kg) (Corbet et al., 2016). Therefore,
585 the dose of siRNA must be adapted to the used system. That means that it is not the use of more
586 siRNA that increases the silencing efficiency of the nanovector as shown in these two following
587 studies. Ragelle *et al.* showed a knockdown of targeted gene expression (GFP) of 80% using their
588 siRNA nanovector composed of three polymers: PEG, chitosan and PEI, and functionalized with RGD
589 peptide in GFP model cells, at a siRNA concentration of 100 nM (Ragelle et al., 2015). However,
590 Veiseh *et al.* used more than twice as much siRNA in a nanovector based on SPION, PEGylated
591 chitosan and PEI and functionalized with a tumor-targeting peptide to obtain a GFP silencing
592 efficiency of 62% (Veiseh et al., 2010).

593 **3.3.3.2. Treatment time and administration protocol**

594 For an efficient siRNA transfection *in vitro*, it is important to consider a sufficient treatment time,
595 long enough for the internalization of the siRNA nanovector (Table 4). As an example, Cavalieri and
596 colleagues exposed PC-3 cells to a nanovector prepared with anti-survivin siRNA for 72 h. After this
597 treatment time, they obtained a negligible down-regulation of the protein survivin (<10%). The
598 increase of the incubation time of siRNA nanovector with cells from 72 h to 120 h resulted in a
599 marked silencing in the targeted gene (almost 60 %) (Cavalieri et al., 2015). Similarly, in a previous
600 study published by our team, it was shown that the optimization of the treatment time of MDA-MB-
601 231 cells expressing GFP with CS-MSN could improve the inhibition efficiency of the expression of
602 GFP. An increase of the silencing of the targeted protein up to 4 h of treatment and the prolongation
603 of this time did not improve the efficiency (Ben Djemaa et al., 2018). For *in vivo* studies, the
604 treatment time can be translated by the administration protocol (i.e. number of injections and
605 interval between injections). Many administration protocols with different numbers of injections and
606 different administration schemes were described in the literature (Table 5). Tingjie *et al.* injected a
607 siRNA nanovector 17 times (every other day for 34 days) (Yin et al., 2016). However, Werfel and

608 colleagues administered their siRNA nanovector twice with an interval of 24 h (Werfel et al., 2017). In
609 both studies, they obtained almost 60% of efficiency.

610 **3.3.3.3. Routes of administration**

611 The choice of the administration route depends on the accessibility of the tumors. In fact, for the
612 tumors with deep localization such as liver cancer (Xie et al., 2014; Zhu et al., 2015), the only way to
613 get access to them is through intravenous administration. However, it is possible to use both
614 systemic or local administration (intravenous or intra-/peri-tumoral (Liu et al., 2011)) for the easy to
615 access tumors such as breast cancer. For the intravenous administration, siRNA nanovectors have to
616 overcome all biological barriers described in section 2.1. However, by using the intratumoral injection
617 the nanovector is directly administrated into the tumor and only the cellular barriers needed to be
618 overcome. Various routes of administration depending on the cancer type have been used (Table 5).
619 In research from Xie and coworkers, the intravenous injection of siRNA at 1.2 mg/kg loaded in a
620 nanovector composed of polymers and calcium phosphate core, in a xenograft liver cancer model
621 showed an inhibition of approximative 60 % in tumor growth (Xie et al., 2014). As an example of local
622 treatment, the intratumoral administration of vectorized siRNA at 250 pmol was applied by Liu *et al.*
623 in xenograft breast cancer model for *in vivo* evaluation of the down-regulation efficiency of
624 luciferase. Results showed a significant reduction of the luciferase expression in the tumor (Liu et al.,
625 2011). Yet, for the treatment of xenograft carcinoma mouse model, Corbet and colleagues used both
626 intravenous and peritumoral route to deliver a combination of vectorized therapeutic siRNA. This
627 treatment led to a dramatic tumor growth inhibition (about 60%) upon peritumoral but also systemic
628 administration.

629 **4. Summary and concluding remarks**

630 In summary, an interesting approach to overcome the extra- and intracellular barriers for the
631 delivery of naked siRNA is the use of electrostatically assembled polymer-based nanovectors. One

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

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

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

649

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655 **Conflicts of interest**

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

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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	(Cavaliere 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	MCMPCFTTDHMARKCDDCCGGKGRGKCYGPQCLCR	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
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studies				
Receptor-mediated endocytosis	NP-siRNA-CTX	Fluorescence microscopy Endosomal integrity assay: calcein	Nanovectors are able to escape endosomes	(Veisoh 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	Decrease of the colocalization of nanovectors		(Cavalieri et
	Confocal microscopy	with lysosomes after 24 h		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-lysine

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	GFP	No data	8	68	(Veisheh et al., 2011b)
NP-siRNA-CTX	SPION, PEG, chitosan, PEI, chlorotoxin, siRNA	TC2 C6	GFP	225 nM	2	62	(Veisheh et al., 2010)
NPEG-PLLs	NPEG-PLL, siRNA	PC-3	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, 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	(Veisheh et al., 2011b)

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

Nanovector	Composition	Model	Target gene	siRNA dose	Administration		Silencing efficiency (%)	Reference
					protocol (number of injection)	Administration route		
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_1 α	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

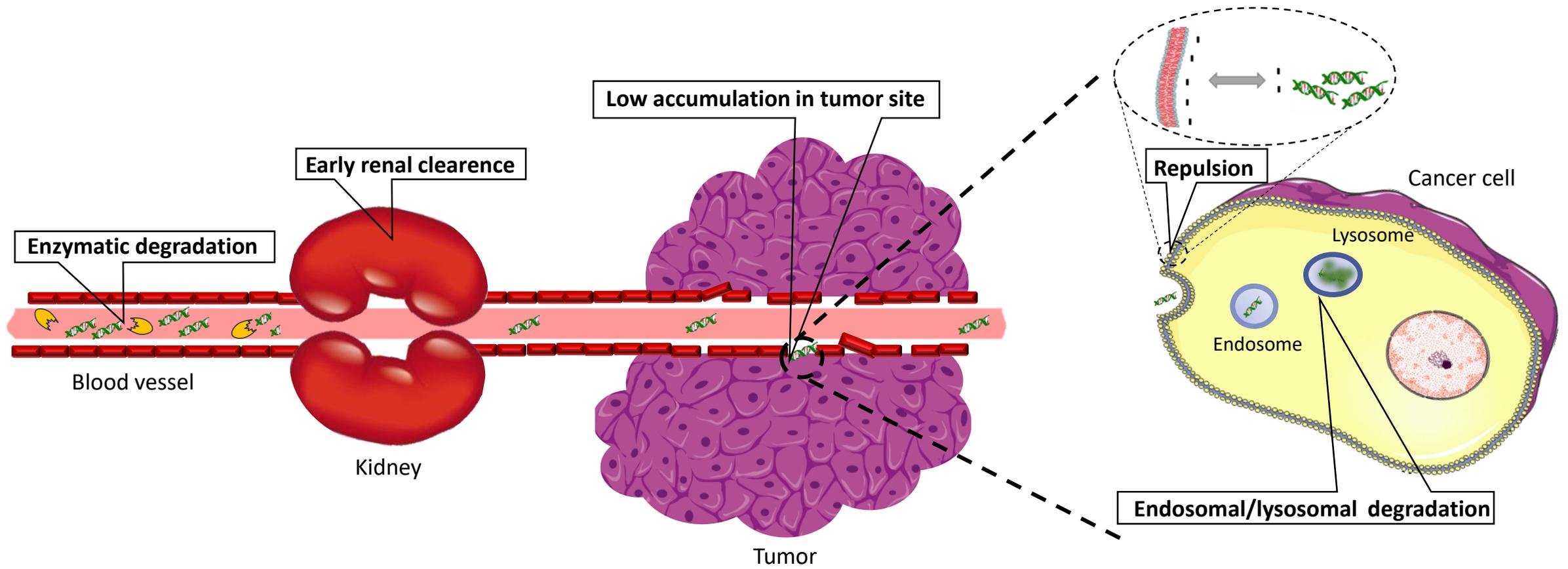
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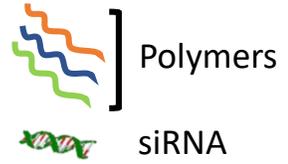
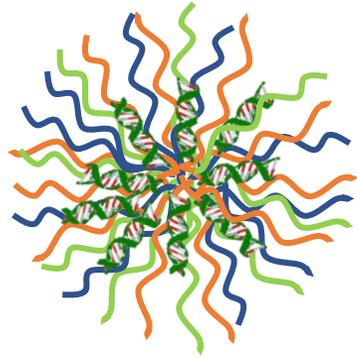
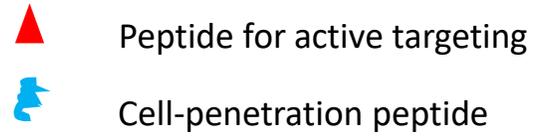
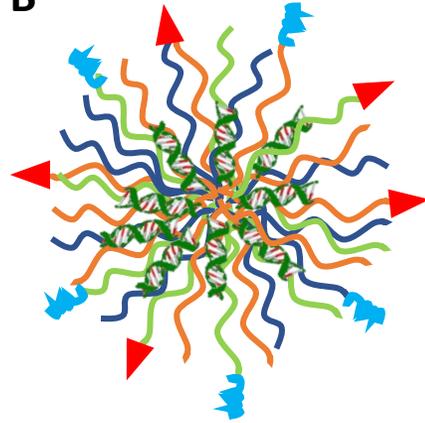
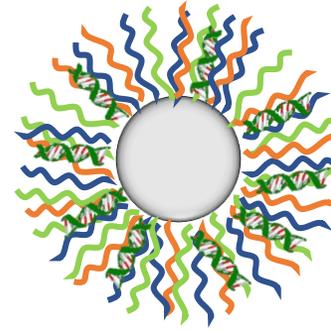
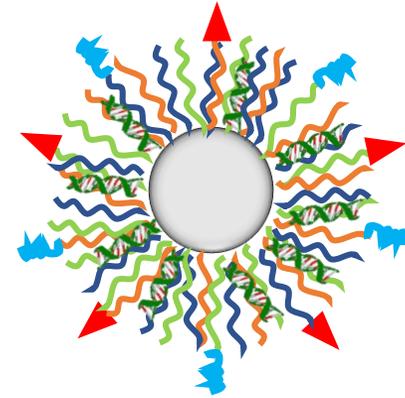
Figure 1. Illustration of extra- and intra-cellular biological barriers for siRNA-based cancer therapy. Extracellular barriers: enzymatic degradation in the blood, early elimination by the kidney, low accumulation in the tumor site, and repulsion at the surface of the cell membrane. Intracellular barriers: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

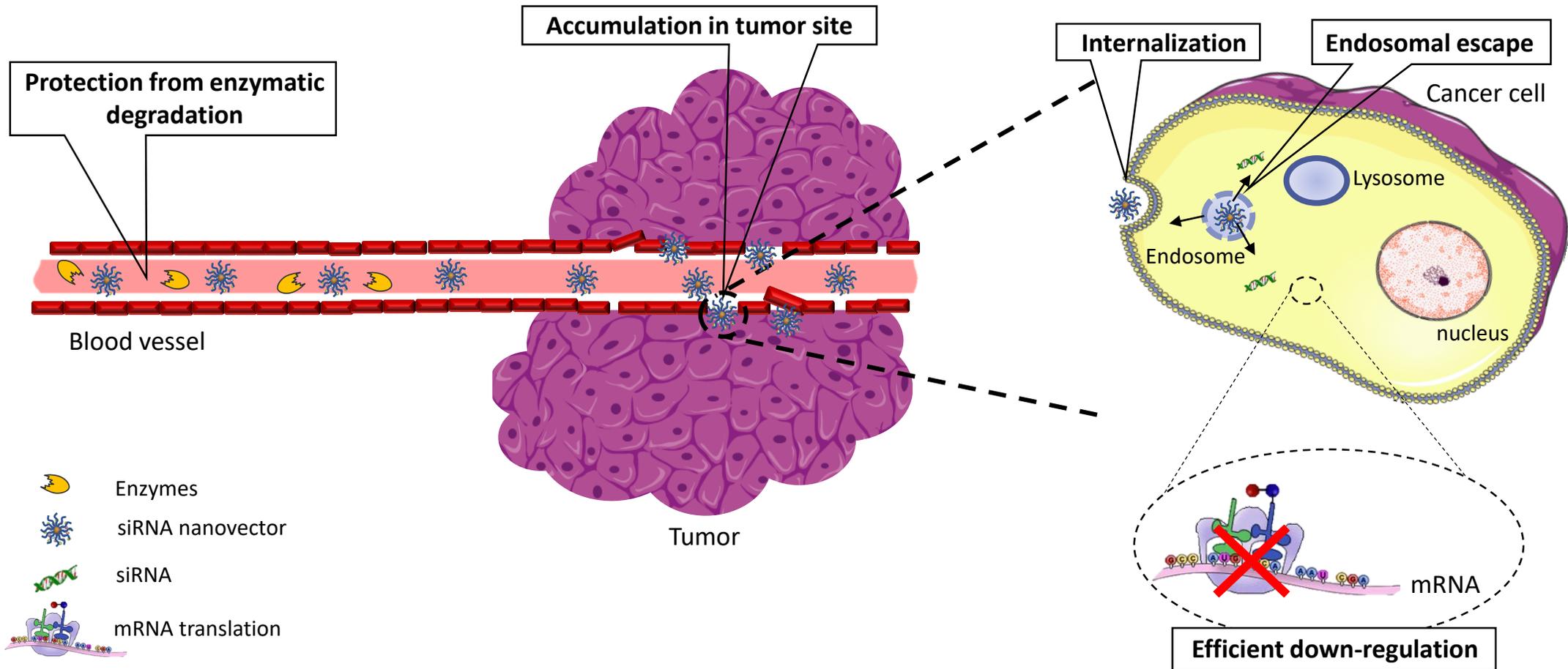
Figure 2. Schematic presentation of electrostatic assembled polymer-based siRNA nanovectors (EPSN): (A) containing only polymers and siRNA, (B) decorated with peptides, (C) containing an inorganic core, and (D) containing an inorganic core and decorated with peptides.

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.

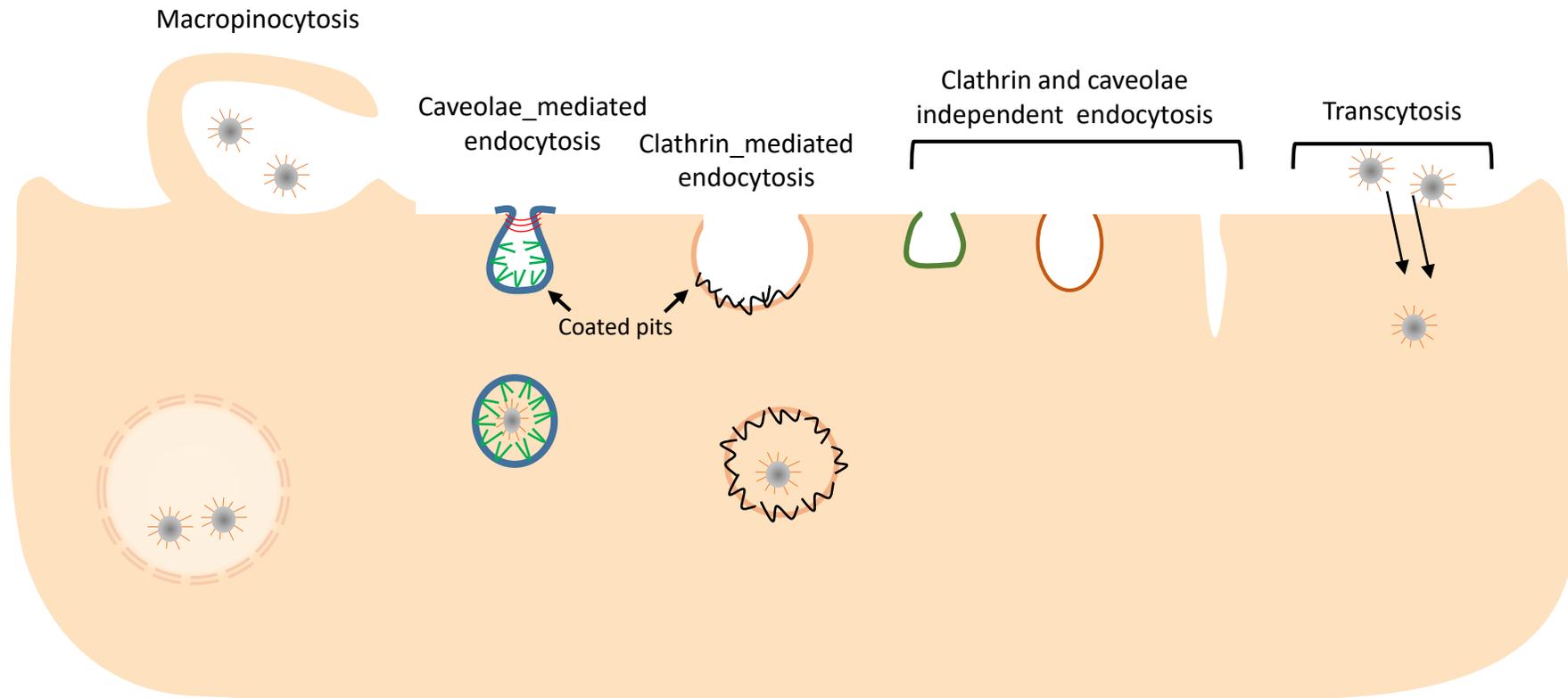


A**B****C****D**



Endocytic pathway

Non-endocytic pathway



 Nanovector

 Caveolin 1

 Clathrin

 Dynamin

