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Review article

# Polymer nanocarriers for the delivery of small fragments of nucleic acids: Oligonucleotides and siRNA

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ABSTRACT

The success of the application of new therapeutic methods based on RNA interfering strategies requires the *in vivo* delivery of active ODN or siRNA down to the intracellular compartment of the target cells. This article aims to review the studies related to the formulation of RNA interfering agents in polymer nanocarriers. It will present the different types of polymer nanocarriers used as well as the biological activity of the resulting ODN and siRNA loaded nanocarriers. As will be explained, the part of the *in vitro* studies provided useful data about the intracellular delivery of the formulated RNA interfering agents. Investigations performed *in vivo* have considered animal models of different relevant diseases. Results from these investigations have clearly demonstrated the interest of several polymer nanocarriers tested so far to deliver active RNA interfering effectors *in vivo* making possible their administration by the intravenous route.



## 1. Introduction

Gene down-regulation is a critical research area in life science. It consists in the reduction of the gene transcription. This approach is widely used in the fundamental research as a rapid method to identify and to characterize the function of known-genes. Results coming from these researches can directly be applied to identify new target genes involved in disease processes. Indeed, several severe diseases including many types of cancer result in the abnormal expression of defined genes. Thus, the comprehension of genetic mechanisms involved in such pathological processes can be very useful for the development of new drugs based on a gene inhibition expression effect.

The down-regulation of a gene can be achieved either at the transcriptional level or at the post-transcriptional level. The effectors acting on the post-transcriptional level are small nucleic acids including small interfering RNA (siRNA) and antisense oligodeoxynucleotides (ODN). They are able to recognize the target mRNA with a high specificity through a base pairing complementary sequence process resulting in the degradation of the target mRNA by intracellular nuclease. This process was named RNA interference (RNAi).

Application of RNAi was first investigated as a novel approach to control virus infections [1]. It was shown that the intracellular proliferation of viruses can be stopped by inducing a specific down-regulation of the expression of the viral genes in infected cells by targeting the corresponding mRNA with ODN. Since this first report, research on RNAi has been widely extended with the aim to discover novel drugs for the treatment of other diseases [2]. For several reasons, cancers are very good candidates for the development of treatments based on RNAi methodology. Cancer cells show abnormal gene pattern. The disease process involves the abnormal overexpression of defined genes including normal and mutated genes and treatments need to be developed on the basis of a high degree of specificity to avoid side effects. Thus, the easiest and more specific approach includes the development of a strategy based on the down-regulation of mutated genes expressed by cancer cells. Other target genes to be down-regulated in cancer disease can be a part of the normal genes like the VEGF gene implied in the proliferation of the blood vessels in the tumor tissues. Independently from the chosen strategy, the major obstacles to face when developing ODN or siRNA as drugs are to keep their biological activity intact *in vivo* and to help them to reach their target mRNA in the cell cytoplasm. Indeed, by themselves, short nucleic acids are totally inactive *in vivo* if they are not protected against nuclease degradation and if they are not conveyed

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down to the cell cytoplasm where their biological targets are located [3,4]. Therefore, their application in medicine remains sustained to the finding of suitable delivery methods improving both their resistance to nuclease degradation and their intracellular penetration. Chemical modifications were believed to resolve the problem of nuclease degradation. Although this approach actually improved ODN and siRNA stability in biological media, it was found that it reduced interfering specificity and efficiency. Additionally, this strategy was not able to resolve the problem of intracellular penetration [5–7]. On the contrary, nanotechnologies present a much better profile to insure both protection against nuclease degradation and improvement of intracellular targeting. Such benefits have been proved for many types of drugs [8] and were also found to apply efficiently for the delivery of nucleic acids as it will be discussed in this review.

## 2. RNA interference mechanism (RNAi)

RNAi includes strategies that result in the down-regulation of the expression of a target gene at the post-transcriptional level using RNAi agents. Highly specific RNAi agents were identified among two types of short chains nucleic acids: ODN and siRNA. Although, the biological activity of these molecules results in the inhibition of the synthesis of the protein encoded by the target gene, the intimate mechanism by which gene expression is down-regulated depends on the nature of the interfering agent. This part of the review summarizes the RNAi mechanisms of ODN and siRNA.

### 2.1. Oligodeoxynucleotides (ODNs)

ODNs are short single stranded DNA fragments which were initially developed to correlate DNA sequence with the corresponding mRNA [9]. In this aim, Paterson et al. showed that a fragment of DNA with a complementary sequence of a targeted mRNA was able to stop the protein translation process in a very specific manner. Only a year later, Stephenson et al. [1] and Zamecnik et al. [10] showed that such DNA fragments could be used to inhibit the development of a virus in viral infected chick embryo fibroblasts. Since then, approaches using ODN to inhibit protein synthesis of a targeted mRNA were successfully observed in many occasions [11]. Translation arrest by ODN was found to be length and concentration dependant. For instance, effective ODN sequences are typically composed of 15 to 30 bases. They are able to form heteroduplexes with the targeted mRNA after sequence specific annealing (Fig. 1). It was shown that several ODN sequences complementary to different parts of the mRNA could be used to stop the translation process of a specific protein, resulting in a reduction of the concentration of the protein coded by the targeted mRNA in the cell [12,13].

At the moment, there are two hypotheses that received much attention to explain the mechanism by which ODNs are down-

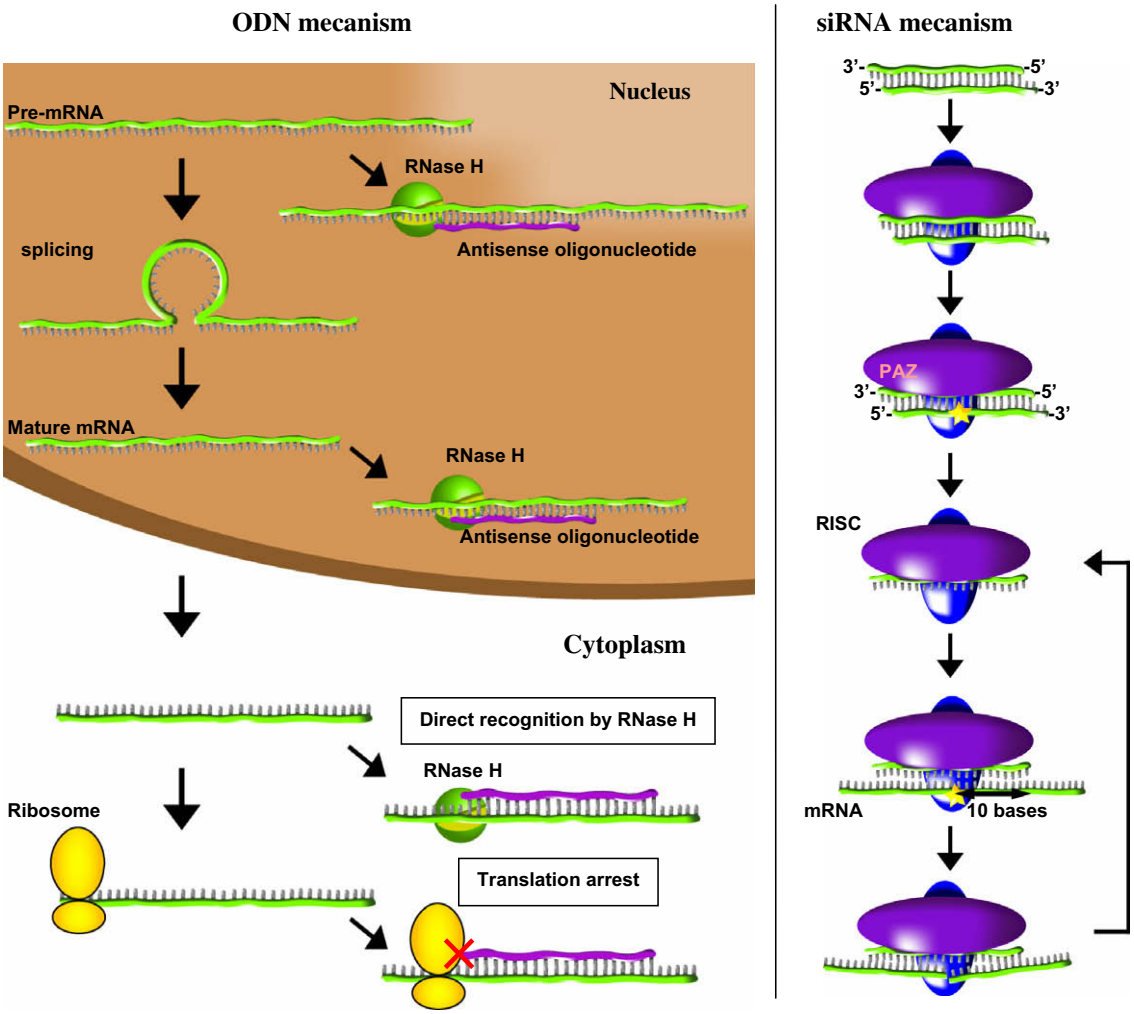



**Fig. 1.** RNAi mechanisms involving ODN and siRNA.

regulating the expression of the targeted protein (Fig. 1). In the first, ODNs directed towards the mRNA are thought to arrest protein elongation by a steric hindrance effect interfering with the mechanisms of translation involving ribosomes. Indeed, it is believed that the 40S ribosomal subunit is blocked by the heteroduplex which obstructs the progression of the ribosome along the mRNA. This is mainly observed when the ODN targets the 5′ untranslated region of the mRNA [14]. The hypothesis proposed in the second mechanism is based on the action of the RNAse H. The RNAse H was discovered by Minshull and Hunt 20 years ago [15]. In the cells, this enzyme is responsible for the degradation of the Okasaki's fragment during DNA polymerization where it is able to recognize DNA–RNA heteroduplexes and induces a specific degradation the RNA strand [16]. Thus, ODN–mRNA heteroduplexes are ideal substrates for the enzyme, leading to the degradation of the mRNA hence translation arrest [17,18].

### 2.2. siRNA

The siRNAs consist in 19–23 double stranded ribonucleotides with two nucleotides overhang at the 3′ ends and phosphates groups at 5′ ends [6,19–21]. Their mechanism of action was elucidated recently thanks to the comprehension of the function of the Argonaut protein family [22,23]. In human cells, one of the strand, named the guide strand, is recognized by the RNA-induced silencing complex (RISC), in which the main actor is an Argonaut protein (hAGO2 in human) [24]. The protein hAGO2 contains four domains, including a PAZ domain (Protein Argonaute Zwille domain) which is able to recognize the 3′ single stranded end of the siRNA, and a PIWI domain (P-element-Induced WImpy testis) able to recognize the 5′ phosphate end of the siRNA and possessing a RNase H-like site for the catalytic degradation of the mRNA [25,26]. Due to favorable thermodynamic conditions, only the guide strand of the siRNA interacts with RISC. The 5′ end of the siRNA which is the less stable will be incorporated in RISC and subsequently the second siRNA strand, the passenger strand will be degraded [24,27]. The guide strand retained by RISC serves as a template for the specific recognition of the targeted mRNA according to complementary base-paring mechanism. From this step, two post-transcriptional events can occur. When the guide strand matches exactly with the mRNA, RISC cuts the mRNA between the two bases apparied to the tenth and the eleventh nucleotides from the 5′ end of the siRNA, This "Slicing mechanism" can be repeated several times inducing mRNA degradation by cytosolic exonuclease. The second mechanism generally occurs when the guide strand presents mismatches regarding the mRNA sequence. It is mediated by one of the AGO subfamily member of the Argonaut proteins (hAGO1–4). This mechanism named "bypass mechanism" is not followed by mRNA slicing but induces translation arrest. It is known to occur in cell foci involved in the turnover of mRNA termed P-bodies [22,26]. In summary, the double stranded RNA interfering effectors mediate mRNA degradation before it is translated into protein by a complementary sequence recognition mechanism.

In contrast with plants and worms, mammalian cells lack of amplification mechanism by RNA dependant RNA polymerase protein, leading to a dilution of the RNAi molecule after each cell division. Thus, to obtain a complete silencing of the targeted gene over a long period of time, several administrations of the interfering molecules are needed. It can be noted that double strand RNA longer than those of the optimal length of a siRNA, i.e. 27 base pairs, are no longer used to produce interfering RNA effect. Indeed, they induce unwanted cell apoptosis in mammalian cells through the activation of interferon via the Protein Kinase-R pathway [19,26,28,29].

## 3. Design of polymer nanotechnologies for the delivery of RNAi agents

Fig. 2 illustrates the schematic structure of different nanocarriers used for RNAi delivery. In general, nanocarriers are loaded with RNAi effectors by ionic interactions between the negative charges of the nucleic acid phosphate groups and a polycation, resulting in the formation of a polyelectrolyte complex. Cationic polymers were initially used for gene delivery but systems were optimized to suit with the delivery of RNAi agents [30,31]. These polymers include poly(ethyleneimine) (PEI), poly-(L-lysine) (PLL), chitosan, gelatin, poly(dimethylaminoethylmethacrylate) (PDMAEMA) and poly(trimethylaminoethyl-methacrylate) (PTMAEMA). Other loading strategies include covalent binding of nucleic acids onto the nanocarrier surface, or with a hydrophilic polymer like the polyethylene glycol) (PEG) before the formation of polyelectrolyte complex with polycations. The different types of polymers that were used to design nanocarriers are summarized in Table 1. The following part of the review introduces the different systems and the way they are loaded with ODN and siRNA. The simplest nanocarrier is represented by the polyplexes, whereas polymer micelles may take a quite sophisticated structure.

### 3.1. Polyplexes

Thanks to complementary charge annealing, the different polycations (i.e. PEI, PLL or chitosan) can form, with nucleic acids, nanosized polyionic complexes named polyplexes. The main formulation parameter to obtain polyplexes within desired size range is given by the *N*/*P* ratio which is defined as the ratio of the number of amine groups of the polycation divided by the number of phosphate groups of the nucleic acid. Thus, the "*N*" value also corresponds to the number of positive charges involved in the formation of the complex while the "*P*" value gives the number of negative charges. In general, the net charge of the polyplex appears positive when the *N*/*P* ration is above 1. The internal structure of polyplexes can be described as a gel-like structure, in which the complexed polyelectrolyte chains are swollen by water molecules.

PEI is probably the first line polycation proposed to formulate polyplexes with nucleic acids including ODN and siRNA. It can exist as linear or branched polymer. Every three atoms is an amine making PEI a highly protonable polymer which can induce a buffering effect named the "proton sponge" effect [32–34]. Because of this effect, PEI containing nanocarriers can provoke the explosion of the cell endosomes with the consequence to release the nucleic acid in the cytoplasm. This leads generally to a significant transfection efficiency. The mean size of PEI/siRNA polyplexes depends on the *N*/*P* ratio. For instance, polyplexes prepared with the commercial product, jetPEI® using a *N*/*P* ratio of 10, have a diameter of about 40 nm [35]. A *N*/*P* ratio of 2 leads to a bigger size (between 120 and 170 nm) [36]. Noteworthy, the molecular weight of the PEI influences the transfection capability of polyplexes. High

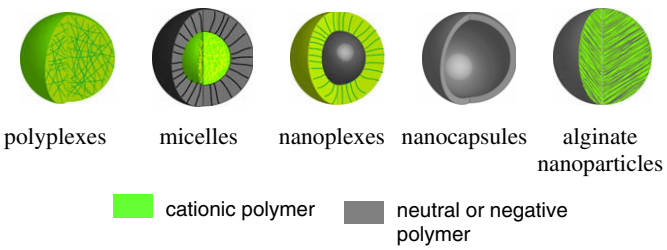
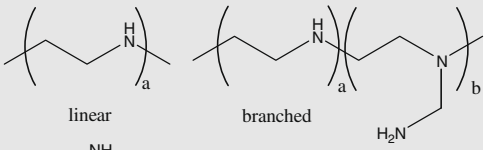
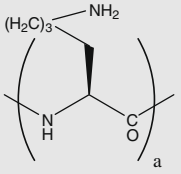
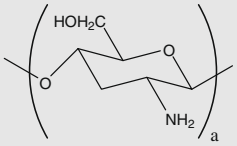
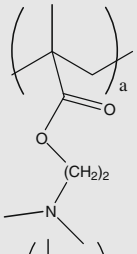
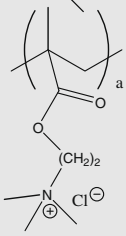
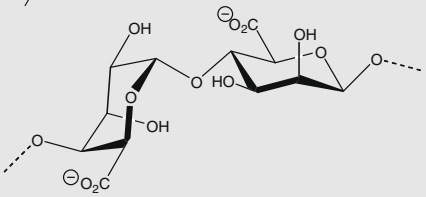
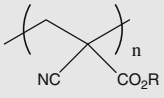
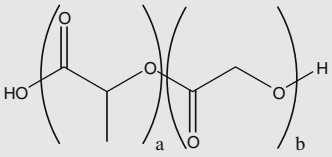
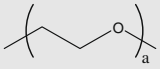
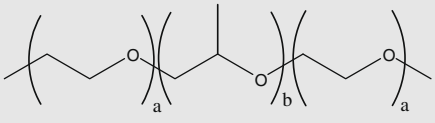



**Fig. 2.** Structure of the different nanocarrier systems.

**Table 1**
Chemical structure of the common polymer used for nanocarrier preparation.

| Type | Name | Formula |
|---|---|---|
| Cationic | PEI |   |
| | PLL |  |
| | Chitosan |  |
| | PDMAEMA |  |
| | PTMAEMA |  |
| Negative | Alginate |  |
| Neutral | PACA |  R=Bu,iBu,iHe |
| | PLGA |  a= number of unit of lactic acid b= number of unit of glycolic acid |
| | PEG |  |
| | Pluronic |  |

molecular weight PEI polyplexes are highly efficient for transfection. However, high molecular weight PEI is also characterized by an important cytotoxicity [37–39]. The toxicity can be reduced by masking the charges but, in counterpart, this also decreases the transfection efficiency [38–40]. Therefore, several authors have proposed to modify PEI according to a different strategy. For instance, ketalization of PEI leads to a dramatic reduction of the polymer's toxicity [41]. The modified PEI becomes labile at a pH around 5.0 and degrades when it reaches the endosome of the cells where it can release the nucleic acid. It was found to improve the compaction of the nucleic acids. In general, a *N*/*P* ratio of 40 is enough to obtain polyplexes with a size around 150 nm. The high compaction of siRNA in such polyplexes is confirmed by the decrease of ethidium bromide incorporation in the nucleic acid entrapped in the ketalized PEI polyplex. To obtain a high degree of compaction, the molecular weight of the PEI must be above 800 Da. Under this value, ketalized PEI becomes inefficient to condense siRNA. The polyplexes formed have a size around 400 nm even for a very high *N*/*P* ratio of 100. In another study, Swami et al. have suggested to use a PEI-bisepoxide in which the primary amines are changed to secondary and tertiary amines [42]. With this modification, the global charge of the PEI remains unchanged while the proton sponge effect is fully preserved. Reduction of the number of the primary amine groups leads to a decrease of the PEI toxicity, providing polymer derivatives which are more suitable for *in vitro* transfection of cells.

Polyplexes prepared with PLL were early tested for ODN delivery. Because of the potential toxicity of PLL, the formation of polyplexes was investigated using glycosylated PLL [43,44]. The size of the nanoplexes obtained with mannosylated and galactosylated PLL was 110 and 160 nm, respectively. Interestingly, such polyplexes prepared with a *N*/*P* ratio above 1 (*N*/*P* = 1/0.6), expected to show a positive net charge, displayed, on the contrary, a negative value of the zeta potential. To explain this result, it was suggested that the ODN was more or less exposed at the polyplex surface [44].

Chitosan can also be used to prepare polyplexes. Different properties of chitosan can dramatically influence the formation of complexes with ODN or siRNA. Thus, Chitosan molecular weight, deacetylation degree, and chitosan to nucleic acid weight ratio are important parameters to control as they influence the resulting charge of the complex formed with the nucleic acid. It has been reported that the cytotoxicity of chitosan was lower than the cytotoxicity of PEI [31]. In addition to its low cytotoxicity, chitosan achieves successful intracellular delivery of intact nucleic acid since it allows nucleic acids to escape from endosomes prior to the occurrence of nuclease degradation [45]. The *N*/*P* ratio influences the final size of polyplexes [45]. Low values of the *N*/*P* ratio (from 2 to 6) lead to polyplexes with size ranging from 328 to 223 nm. Smaller size of 139 nm could be obtained using a higher *N*/*P* ratio of 285. The size of the chitosan-polyplexes is also influenced by the chitosan molecular weight [46]. Chitosan of 110 kDa leads to polyplexes ranging from 150 to 300 nm, whereas a molecular weight of 270 kDa leads to polyplexes with size ranging from 180 to 450 nm. Size of polyplexes prepared with chitosan modified by glutamate was around 180 nm and found independent of both the molecular weight of the chitosan derivative and the *N*/*P* ratio.

Polycations are the main components of polyplexes. Although, they present advantages including a high payload in siRNA and ODN and a very high transfection efficiency, these components are known to interact with proteins and to be rather toxic. These drawbacks have limited so far the development of polyplexes as suitable RNAi effector delivery systems for the application in human therapy. It can be believed that this aspect will continue to hinder the development of these systems in the future.

### 3.2. Polymer micelles

Polymer micelles are prepared from either diblock copolymer AB, or triblock copolymers ABC or ABA. In general, one part of the copolymer, part A, is a polycation including PEI, PLL, PTMAEMA or PDMAEMA [47–49] and the second part, part B, is a highly hydrophilic polymer such as PEG, dextran [50] or PHPMA [48]. It is interesting to point out that PDMAEMA and PTMAEMA are non-biodegradable and relatively toxic polymer, but their use as a part of an amphiphilic copolymer may reduce their toxicity and make them useful for transfection [48,51,52].

The micelles formed spontaneously after mixing the copolymer with the nucleic acid thanks to the occurrence of electrostatic interactions between the nucleic acid and the positively charged block of the copolymer. The core of the resulting micelles is composed by a polyelectrolyte complex with a gel-like structure close to the one found in polyplexes [53]. The corona of the micelles, which is composed of the block B of the copolymer, forms a steric barrier to the aggregation and serves as stabilizer for the polymer micelles. A minimum size of 2 kDa is needed to have a stabilization effect by the PEG [54]. An original approach for the PEGylation of a polycation is based on the use of the adamantane/cyclodextrin affinity since adamantane can form stable inclusion complexes with cyclodextrines. Thus, an adamantane residue grafted on a PEG chain can serve as an anchor to attach the PEG chain to cyclodextrin containing polycation via the formation of an adamantane/cyclodextrine inclusion complex [55] (Fig. 3). The micelles are then formed by complexation of the nucleic acid with the polycationic part of the previous assembly. One advantage of this method is that it did not require the formation of a covalent linkage between the polycation and the PEG. A further advantage of this method is that it allows the mixing of components including an adamantane anchor to adjust the surface property of the micelles. For instance, the authors have designed targeted micelles including the mixtures of adamantane-PEG and adamantane-PEG-transferrin (AD-PEG-Tf) polymers at different compositions to target cells overexpressing the transferrin receptor. This system is therefore very flexible.

As polymer micelles are generally more stable than polyplexes, their size is much smaller. For instance, the diameter of polyplexes obtained with PEI/siRNA (120 nm) is reduced to 90 nm in the corresponding polymer micelles formed with PEG-PEI/siRNA which are stabilized with PEG [36]. The same tendency was also reported for the polymer-adamantane/cyclodextrin micelles [55]. PEGylation of the polycation leads to micelles with a size below 100 nm whatever the formulation was, whereas the size of unPEGylated polycation complexed with siRNA depended closely on the formulation and generally exceeded 150 nm in diameter. In parallel, the zeta potential of the polyelectrolyte complex formed between PEI and siRNA using a *N*/*P* ratio of 2 which is highly positive (+35 mV) was reduced to +5 mV in the corresponding PEG-PEI/siRNA micelles. This can be explained by the fact that the PEG chains located at the micelle surface may shield the net charge of the polyelectrolyte complex constituting the core of the micelle.

In general, polymer micelles have a spherical shape. Their stability depends on the nature of the polycation moiety of the copolymer [56]. Long polycation chain increases the micelle size. For instance, with PEG-PLL copolymers, the diameter of the micelles varies from 23 nm for PEG(5 kDa)-PLL (18 kDa) to 37 nm for PEG(5 kDa)-PLL(78 kDa) [47]. Similar behaviour of the micelles was found with PEI containing copolymers [47,57]. The relation between the hydrophilic polymer length and the micelle's diameter remains, however, unclear and no general feature can be drawn. Interestingly, the size of the nucleic acid has no influence on the final diameter of the micelles [47].

Recently, an alternative route was proposed to obtain polymer micelles using PEG directly conjugated to nucleic acid instead of PEG-polycation copolymers [57,58]. In this case, the nucleic acid, either ODN or siRNA, was included as a part of the copolymer. The addition of a polycation forms the polyelectrolyte complex corresponding to the core of the micelle. In this case, the linkage between the polymer and the nucleic acid has to be biodegradable to allow the release of the nucleic acid in the intracellular medium of the targeted cell. Different linkages were already investigated, i.e. amide linkage degraded by amidases [58], disulfide linkage degraded by the glutathione [59,60] or ester linkage cleaved by esterases at the low endosomal pH [61]. The resulting micelles had a very narrow size distribution, but in general a slightly bigger size than the corresponding micelles obtained by hydrophilic polymer-polycation copolymers complexed to nucleic acid. For instance, PEG-ODN/PEI, PEG-ODN/KALA and PEG-siRNA/PEI micelles were shown to have a diameter of 70 nm [57,58,62], whereas PEG-PEI/ODN, PEG-PLL/ODN or PTMAEMA-PHPMA/ODN micelles showed a diameter of 40 nm [48,49,63]. In PEG-nucleic acid/KALA

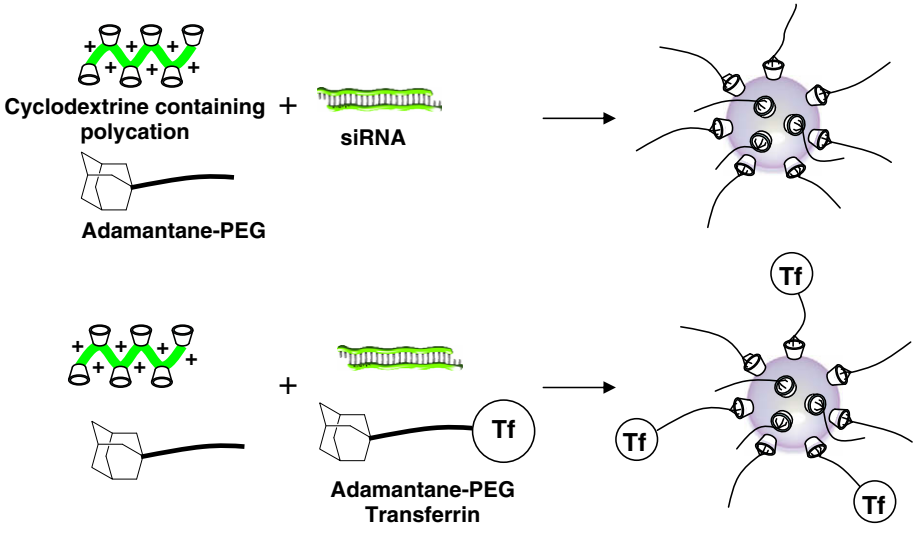



**Fig. 3.** Formation of micelles by complexation of the nucleic acid with polycation containing cyclodextrin complexed with adamantane-PEG and adamantane-PEG-transferrin.

micelles, where the KALA cationic peptide was used to form the core of the micelles, the molecular weight of the hydrophilic polymer had a high influence on the final size of the micelles [62]. An increase of the diameter from 70 to 200 nm was reported for an increase of PEG molecular weight from 2 to 5 kDa [59,62]. One advantage of using PEG-ODN or PEG-siRNA copolymers is that micelles can form even with low molecular weight polycations which are less toxic. Thus, the micelles obtained with these new ODN-PEG conjugates are expected to be better tolerated by living animals than the first generation of nucleic acid loaded onto polycation containing copolymers.

A very interesting study has combined Pluronic F127 chemically linked to PEI 2 kDa with a PEGylated siRNA. The sonication of the PEI-Pluronic copolymer let to in the formation of micelles prior to the addition of the PEGylated siRNA [64,65]. The term of "nanocapsule" used by the author remains to be further clarified as the composition and the biological behaviour suggest a micelle type organization. Interestingly, the adsorption of the PEGylated siRNA instead of naked siRNA resulted in a higher stability of the micelle-like polyplex. The complex appeared, indeed, more stable in a competition assay performed with heparin. These "micelles" were characterized by a size of 119 nm (at 37 °C) which increased up to 412 nm at 15 °C leading to 42-fold increases in the volume. This swelling property is interesting because it can be used to make possible the escape of the nucleic acid from the endosomes after endocytosis by cells. In this case, the micelles can be endocytosed by the cells at 37 °C and the application of an external cold shock at 15 °C can induce the release of the nucleic acid in the cell cytoplasm thanks to the swelling of the nanocarrier.

Zhang et al. have suggested to further crosslink the shell of the micelles to improve their stability in high ionic strength conditions [52]. Hence, ODN containing micelles formed with a copolymer PEG-methacrylate-PDMAEMA were further polymerized using ethylene glycol dimethacrylate. The size of the resulting shell-crosslinked micelles was smaller than the size of the corresponding uncrosslinked micelles. As expected, the crosslinked micelles, named "knedels", appeared to be very stable even at a high ionic strength. These crosslinked micelles displayed a structure which resembled to nanocapsules.

Despite the many efforts made to formulate polymer micelles with small polycations or with modified polycations to reduce the toxicity of these compounds, they remained cationic species with their potential inherent toxicity. Therefore, it is unlikely that the formulations of such polymer micelles will pass the barriers of the health authorities to be developed as suitable carriers for the *in vivo* delivery of siRNA and ODN in human.

### 3.3. Nanoplexes

The word "nanoplexes" was suggested to design preformed matricial systems having the nucleic acids bound on their surface. In most of the cases, the nucleic acid was loaded via ionic interactions on the positively charged nanocarrier surface. Recently a new approach was also proposed involving the avidin–biotin interaction.

Several types of nanoparticles made of poly(alkylcyanoacrylate) (PACA) have been proposed as suitable drug delivery systems for ODN and siRNA delivery [66]. They were obtained by in situ emulsion polymerization of the corresponding monomer. Initiation of the PACA polymerization on a polysaccharide leads to amphiphilic copolymers which stabilize nanospheres by exposing the polysaccharide moiety at the nanosphere surface [67–70]. For instance, PACA nanoparticles prepared according to the original anionic emulsion polymerization of Couvreur et al. [71] are coated with dextran which gives the nanocarriers a slightly negative zeta potential. The mechanism of polymerization is set by the experimental conditions (pH, presence or absence of cerium IV) [72]. This is noteworthy because it will define the final structure of the PACA nanoparticles, and in turn, their properties. By anionic polymerization, several PACA chains are grafted on a single polysaccharide molecule giving a graft copolymer. The polysaccharide chains take a conformation of loops and trains at the nanoparticle surface. On the contrary, in the case of radical polymerization, only one chain of PACA is grafted on one chain of the polysaccharide giving a linear block copolymer. In this case the polysaccharide chains form a brush like structure at the nanoparticle surface. The size of the nanocarriers depends on the nature of the monomer with poly(isobuthylcyanoacrylate) nanoparticles (170 nm) being smaller than poly(isohexylcyanoacrylate) nanoparticles (200 nm). To achieve nucleic acid association on those nanocarriers via the formation of ion-pairs, it was suggested to invert the surface charge. This was done by adsorbing a positively charged surfactant onto the nanocarrier surface. In this view, surfactants with different carbon chain lengths hence having different hydrophobic character were investigated, i.e. dodecyl, tetradecyl or cetyltrimethylammonium bromide (DTAB, TTAB or CTAB). The surfactant with the longer carbon chain, CTAB, was the most efficient to promote the adsorption of ODN on the nanoparticle surface [67]. It was assumed that it provided the more stable anchor for the positive charge at the nanocarrier's surface insuring also the best stability for the ion-pairs between the ODNs and the cationic surfactant. However, CTAB is known to be teratogenic and toxic meaning that the use of these nanoplexes in human therapy is compromised for further development [73,74].

Chitosan, a polysaccharide from natural sources, includes lots of amine groups in its structure. It generally shows a good biocompatibility and for this reason it appears as a good candidate to replace CTAB on the surface of PACA nanoparticles [75,76]. Chitosan can easily be grafted at the surface of PACA nanoparticles during the emulsion polymerization applied to prepare the nanocarriers [77–79]. The two types of polymerization can be applied leading to the positively charged nanoparticles that are able to complex either siRNA or ODN [79–82]. PIBCA nanoparticles prepared by anionic polymerization are characterized by a diameter of 60 nm, while the nanoparticles prepared by radical polymerization show a diameter of 80 nm. Complexation of siRNA onto the nanoparticle surface led to an inversion of the zeta potential of the resulting nanoplexes [82].

Apart from PACA nanoparticles, poly(lactide-co-glycolide) (PLGA) nanoparticles coated with chitosan were designed as other carriers for chemically modified ODN [83]. In this case, the nanoparticles were prepared by an emulsion–diffusion–evaporation method. The PLGA dissolved in ethyl acetate was added dropwise to an aqueous solution of chitosan under strong stirring. The size and charge of the resulting nanoparticles could be tailored by controlling the parameters of the preparation process. For instance, nanoparticles with size as small as 130 nm can be synthesized using a small amount of poly(vinyl alcohol) (1 mg/mL). The charge of the nanoparticle surface increased with the amount of chitosan and the organic phase/aqueous phase ratio used during the preparation. These nanoparticles could associate chemically modified ODN at their surface.

Gelatine nanoparticles obtained from gelatine type A were found interesting to deliver gene *in vivo*, and were tested for their capacity to be loaded with ODN [84]. These nanoparticles were prepared by a two steps desolvation process. The dehydrated protein nanoparticles obtained are reticulated with glutaraldehyde. Quaternalized amine molecules such as cholamine can be grafted on these nanoparticles to obtain gelatine nanoparticles with a positively charged surface and a diameter ranging from 182 to 278 nm [85]. ODN can adsorb onto the surface of these nanoparticles after 2 h incubation. However, the amount of ODN which adsorbed on the gelatin nanoparticles was greatly influenced by their zeta po-

tential. For instance, it was reported, that a minimum zeta potential of +13 mV was required to efficiently load the nanoparticles with ODN in saline buffer conditions, while the loading efficiency in a highly purified water was totally independent on the zeta potential of the nanoparticles. In PBS or in 0.9% NaCl, a decrease of 40% in the siRNA loading was observed with nanoparticles having a zeta potential of +17.5 mV. However, once the nanoplex was formed, the ODN loading remained unchanged by increasing the ionic strength of the medium.

The use of avidin–biotin interaction is another method for the loading of ODN onto the surface of gelatin nanoparticles. In this case, the surface of the gelatin nanoparticles was thiolated using Traut's reagent. Activated neutravidin reacting with thiol groups could be covalently grafted on the surface of the gelatin nanoparticles [86–88]. Then, biotinylated ODN could be associated with the nanoparticles through the avidin/biotin interaction. The further development of gelatine nanoparticles as they are formulated today can be compromised because of the use of glutaraldehyde as a reticulant agent. This component is known to be very toxic *in vivo* and the health authorities will unlikely deliver authorization on formulations including nanoparticles prepared with glutaraldehyde. New reticulant agents need to be developed before such formulations may have a chance to appear in clinics. Additionally, cationic compounds should be avoided from these formulations because they can generate toxicity problems as discussed above in the case of polyplexes or polymer micelles.

### 3.4. Nanocapsules

Nanocapsules are vesicular type polymer carriers. They are composed of an aqueous core surrounded by a thin polymer envelope. In general, the nucleic acid is entrapped in the aqueous core of the nanocapsules. One consequence of such method of encapsulation is that the release of the entrapped ODN or siRNA only occurs when the nanocapsule wall is biodegraded. Thus, this formulation allows a very good protection of the nucleic acid against metabolization because they are completely isolated from the nuclease rich environment surrounding the nanocapsules *in vivo*.

ODN loaded PACA nanocapsules are synthesized by interfacial polymerization of the corresponding monomer in a water-in-oil nanoemulsion, and proceed according to an anionic polymerization initiated on the water [66]. The aqueous phase contains the nucleic acids and the oily phase contains the monomer [89–92]. In practice, the nanoemulsion is prepared without the monomer which is added once to the nanoemulsion shows the desired characteristics. The polymerization occurring at the oil–water interface leads to the formation of nanocapsules with a mean diameter of ca. 300 nm [89,92]. The technique of the preparation of the nanocapsules was recently improved by Hillaireau et al. who suggested to use a blend of surfactant to improve the colloidal stability of the nanocapsules in aqueous media [93,94]. A reduction of the nanocapsule size down to 250 nm was obtained but a smaller subpopulation of particles with a diameter of 20 nm still remained [89,93]. A certain improvement of the encapsulation rate of ODN can be obtained by coencapsulating a positively charged polymer (either chitosan 30 kDa or branched PEI 25 kDa) [94]. These nanocapsules show several advantages regarding nanoplexes or other drug nanocarrier formulations for the delivery of small nucleic acid fragments. They allow a high payload in ODN or siRNA for a much lower amount of polymer compared with the other systems. Moreover, the incorporation of cationic compounds in their formulation to allow association of nucleic acid is not necessary and they display a neat negative charge of the surface. Among all the delivery systems presented so far, the nanocapsules appear to be the more biocompatible drug carrier for ODN and siRNA. For all these reasons, today they are believed to be one of the most relevant systems to achieve *in vivo* delivery of ODN and siRNA. It should not be surprising that they will be proposed to undergo clinical developments for the administration of RNAi effectors in the future. However, to achieve this goal, the method of fabrication of the nanocapsules will need to be scaled up and the remaining problems about the colloidal stability of the nanocapsules in aqueous dispersions should definitively be resolved.

PLGA nanocapsules were obtained from a double emulsion by solvent evaporation [95]. At first, a water-in-oil emulsion was prepared with Tris–EDTA buffer containing the ODN and a volatile organic solvent – i.e. chloroform or methyl dichloride – containing PLGA. In this preparation, the ultrasounds were used to produce dispersed droplets of very small size. Secondly, the double emulsion was obtained by adding the first emulsion to a poly(vinyl alcohol) solution under sonication. The evaporation of the organic solvent from the double emulsion induced the precipitation of the PLGA around the nucleic acid containing inner water phase allowing the formation of the nanocapsules. The nanocapsule's size was from 279 to 379 nm according to the organic solvent used to dissolve the PLGA [95,96]. The yield of ODN encapsulation also depended on the type of organic solvent used during the preparation of the nanocapsules. For instance, nanocapsules synthesized with methyl dichloride presented a large size (391 nm), and their encapsulation rate of ODN was rather low (1.3%). Interestingly, the coencapsulation of a cationic peptide, Ornithine(10)–Histidine(6) increased the encapsulation rate up to 42% [95]. ODN encapsulation (i.e. 81%) was also improved by replacing methyl dichloride by chloroform [96]. Compared with the previous nanocapsules, these nanocapsules requiring the coencapsulation of polycations to obtain reasonable encapsulation rate of ODN and siRNA will be more difficult to develop at a clinical stage because of the potential toxicity of the polycation. The second drawback of this preparation is the use of chlorinated solvents which will need to be totally eliminated before the use of the formulation for *in vivo* administration.

### 3.5. Alginate nanogels

Alginate are negatively charged, low toxic and low immunogenic polysaccharides extracted from brown algae [97]. The addition of calcium chloride to an alginate solution leads to gelation by ionic cross-linkage of the polysaccharide chains. A mass ratio 6/1 sodium alginate/calcium chloride was identified to allow nanogel preparation [98]. The total concentration of alginate during the calcium gelation, the PLL/alginate mass ratio and the PLL molecular weight were the main parameters to be considered to optimize the size of the nanogels [98–100]. Increase in one of these parameters resulted in the increase of the nanocarrier size. The resulting nanocarriers had a negative zeta potential that increased with the amount of PLL used to formulate the nanogels. The oligonucleotides were loaded inside the matrix of the nanogel by a temperature dependant slow diffusion mechanism taking around 4 days. It was then suggested that the alginate nanogels behave like a sponge. Interestingly, there was no saturation in ODN loading up to a ODN concentration of 10 μmol/L. The loading could be achieved according to two different loading protocols. A "one step protocol" where PLL/ODN complexes were loaded in alginate pregels, or a "two steps protocol" where PLL was first added to form the alginate nanogels followed by the ODN addition. Whatever the protocol used, it was shown that the resulting nanogels had the same properties. Here again, the formulation of the alginate nanogels require the use of a polycation. In more recent works, it was shown that PLL was replaced by chitosan [101], but this does not avoid the use of a polycation in such formulations. Therefore,

the chances of the alginate nanogels to be developed at a clinical level involving a systematic administration of the formulation remain questionable.

## 4. Evaluation of the biological activity of RNAi agents formulated in polymer nanocarriers

The evaluation of polymer nanocarriers for the delivery of ODN and siRNA generally includes two types of studies. The first type of evaluations consists of a set of *in vitro* tests aiming to investigate the protection of the short nucleic acid chain against degradation, the measurement of the intracellular delivery of the nucleic acids in the cells and its *in vitro* RNAi activity. Mechanisms by which ODN and siRNA are delivered into the cells are also investigated. The second type of evaluations includes *in vivo* experiments designed to evaluate the efficacy of the delivery approach and the pharmacological activity of the delivered nucleic acid in relevant animal models. Noteworthy, the development of nanocarriers for ODN and siRNA is a quite recent area of research which has started less than 20 years ago. Although progresses in this field are very rapid, only few systems among those developed so far have yet been evaluated *in vitro* and *in vivo* [102]. This explains why the reader will find, in this part of the review, only a few of the previously presented nanocarriers.

### 4.1. In vitro test

#### 4.1.1. Protection against degradation

The first requirement to make possible the *in vivo* delivery of therapeutically active ODN and siRNA is that the polymer nanocarrier insures a protection against the degradation of the nucleic acid by nucleases [103]. Indeed, it has been proved that there is a strong correlation between nuclease protection efficiency and the level of the gene expression inhibition by a siRNA [54]. Although it is hard to make a direct comparison of the performance shown by the different nanocarriers, because experiments were performed in different conditions depending on the authors, the conclusion can be drawn that, in general, the association of ODN and siRNA to a polymer nanocarrier dramatically improves their stability in the presence of nucleases. While the half-life of free ODN is only a couple of minutes in serum or in cell culture medium, it can be extended up to several hours when associated with a nanocarrier [67,90,103] (see Table 2). Encapsulation inside nanocapsules further improved the protection efficiency against nuclease degradation (31% remained intact after 1 h incubation in foetal calf serum (FCS)) compared with the protection obtained for ODN adsorbed on PACA nanoparticles coated with CTAB (7% remained intact) (Table2) [89]. Large improvement of the stability of ODN was also reported after complexation in polymer micelles. In such formulation, intact ODN was still observed after several hours of incubation with DNAse I or with 10% FCS. The best stability was reported with PEG-ODN/PEI micelles where intact ODN was still observed after 24 h incubation in 10% FCS [61].

Considering siRNA which are more stable in biological media than ODN, the life time can be extended from a couple of hours to 2 or 3 days [50,57] (see Table 2). Chitosan showed a very high protection efficiency for siRNA. Polyplexes prepared with this polysaccharide were able to protect a siRNA for 7 h in 50% FCS, and chitosan coated PACA nanoplexes could even protect siRNA for 48 h *in vivo* in the tumor tissue. Micelles provided a very high protection of siRNA. For instance, intact siRNAs were observed after 3 days in mouse plasma when they were formulated as PEG-PLL/siRNA micelles [50].

#### 4.1.2. Nucleic acid delivery

The second requirement for a successful delivery of RNA interfering agents is that the ODN or the siRNA will be delivered inside the cells where the target mRNA is located. However, due to their extreme hydrophilicity and macromolecular character, siRNA and ODN are unable to penetrate into cells by themselves. Thus, the second important role of a polymer nanocarrier is to help the nucleic acids to reach the cell cytoplasm. It can be an advantage if the polymer nanocarrier shows a positively charged surface which can enhance attractive interactions with the negative charges of the phospholipids composing the cell membrane [104]. Such interactions are essential to promote the intracellular uptake of the drug carrier together with its cargo. This is the reason why most of the systems formulated for the delivery of ODN and siRNA show a positive zeta potential [36,83,105]. Once nanocarriers entered the cells by endocytosis, the nucleic acid needs to cross the endosomal membrane to reach the cell cytoplasm. As already explained before, nanocarriers containing PEI can escape the endosomal com-

**Table 2**
Nuclease resistance of the short nucleic acids formulated in the polymer nanocarriers.

| System type | Composition | Nucleic acid | Conditions | Vectorised nucleic acid | Free nucleic acid | Reference |
|---|---|---|---|---|---|---|
| Polyplexes | Chitosan glutamate 160 kDa deacetylation degree: 86% | siRNA | 5% FBS | Intact 48 h* Fully degraded after 72 h | Intact 24 h Fully degraded after 72 | [46] |
| | | | 50% FBS | Intact 7 h* Fully degraded after 48 h | Immediately fully degraded | |
| Nanoplexes | PIHCA-dextran/CTAB | ODN | DNAse I 5 μg/mL | Intact 15 min* | Fully degraded after 15 min | [67] |
| | PIBCA-dextran/CTAB | ODN | 70% mouse plasma | Intact after 1 h (2%) | Intact after 30 min (2%) | [103] |
| | PIBCA-dextran/CTAB | ODN | 100% FBS | Intact after 1 h (7%) | Intact after 1 h (5%) | [89] |
| | PIBCA-chitosan | siRNA | Inside tumor mice | Intact 48 h* | Intact after 24 h | [82] |
| Alginate | Alginate-PLL (8 kDa) | ODN | 100% FBS | Intact after 1 h (70%) | Fully degraded 1 h | [99] |
| Nanocapsules | PIBCA | ODN | 100% FBS | Intact 1 h (31%) | Intact after 1 h (5%) | [89] |
| Micelles | PEG-PLL/ODN (5–18 kDa) | ODN | DNase I 1.4 U/μL | intact 2 h (60%) | Degraded after 10 min | [47] |
| | PEG–PLL/ODN (5–78 kDa) | | | intact 2 h (90%) | | |
| | PEG–ODN/PEI (4.5–22 kDa) | ODN | DNase I 1.4 U/μL | Intact 2 h (>95%) | ODN-PEG intact after 2 h (40%) | [61] |
| | PEG-PLL/ODN (12–5.6 kDa) | ODN | DNase 0.001 U/μL | Intact 1 h (79%) | Intact 1 h (12%) | [63] |
| | PEG–PLL/siRNA (5–28 kDa) | siRNA | RNase 2 ng/μL | Intact 6 h* | Fully degraded 6 h | [50] |
| | | | Mouse plasma | Intact 72 h* | Few remaining 1 h | |
| | | | Intravenous injected | Intact 2 h* | Intact 1 min | |
| | PEG-siRNA/PEI (5–25 kDa) | siRNA | 50% FBS | Intact 48 h* | Few intact 4 h | [57] |
| | PEG (5 kDa)-siRNA/KALA peptide | siRNA | 5 U Rnase | Intact 10 min* | Few intact | [59] |
| | PEG-PEI/siRNA (550–20 kDa, 25 kDa) | siRNA | 1.2 mU RNase | Intact 30 min* | No data | [54] |

* Only qualitative information available by electrophoresis analyze.

partment via the "proton sponge" effect while nanocarriers containing. PDMAEMA release their nucleic acid content without endosomal disruption [106]. For nanocarriers made with other polycations, the endosomal escape is not fully explained yet.

In many experiments designed to evaluate the intracellular uptake of the nucleic acids delivered by nanocarriers, proteins were removed from the incubation medium during the incubation period of the nanocarrier with the cells. Such an experimental protocol was adopted because it was believed that protein adsorption onto the nanoparticle surface can mask the positive charge of the nanocarriers hence reducing the interactions with the cell membrane [45,46,58,107,108]. Experimental protocols in which serum proteins were added during the incubation period of the nanocarriers with the cells are probably more relevant to anticipate the *in vivo* intracellular uptake of the nanocarrier after intravenous injection [49,57,83,109]. To perform these experiments, ODN and siRNA were labelled with a fluorescent probe (fluoresceine isothiocyanate FITC, or 5-carboxyfluorescein FAM, rhodamine isothiocyanate RITC or Cyanine-5 Cy5) prior to their association with the nanocarrier. The fluorescence-activated cell sorting (FACS) is an interesting technique quantifying the total amount of fluorescent nucleic acid which associates with the cells and to distinguish healthy from apoptotic cells. The quantification can be made on a large sample of cells allowing statistical analysis of the results [58]. Using this method, Kim et al. showed that the *N*/*P* ratio, which is an important characteristic for the formulation of polyplexes, micelles and nanoplexes involving PEI or chitosan, influenced the amount of cell associated with nucleic acid. [109]. For example, the percentage of fluorescent cells using chitosan based polyplexes increased with the *N*/*P* ratio. This result points out the correlation between the positive surface charge of the carrier and the amount of fluorescence due to the nucleic acid which associates with the cells.

FACS provides with global information about the amount of fluorescent nucleic acid which associates with cells whatever the localization, i.e. bound on the cell surface or truly internalized by cells. To investigate in detail how nucleic acid penetrates in the cells and in which intracellular compartment it accumulates, it is necessary to use other investigation methods. Cell imaging using fluorescent confocal microscopy is the most widely used method. Although this methodology is not quantitative, it provides a direct observation of the localization of the fluorescent nucleic acid in the cells giving proofs of cell internalization. This method can also be used to identify the intracellular compartment in which the nucleic acid accumulates. Two types of intracellular distribution of nucleic acid delivered by nanocarriers were reported. When nucleic acids undergo an extensive escape from the endosomal compartment, a diffused fluorescence is observed in the cell cytoplasm or in the cell nucleus [32,45,58,65,91]. In contrast, a punctuated localization of the fluorescence in the cells suggests that the fluorescent nucleic acid accumulated in the endosomes. This is a characteristic for a low endosomal escape [83]. The results obtained by confocal microscopy revealed that PLGA nanocapsules were able to deliver an ODN in primary cultures of dendritic cells only when it was co-encapsulated with the protonable peptide Ornitidine(10)–Histidine(6) [95]. The role of this peptide was to help the nucleic acid to escape from the endosomal compartment, thanks to the "proton sponge" effect. On the contrary, in a previous study performed on smooth muscle cells, a significant intracellular delivery of phosphorothioate ODN was reported without requiring any addition of this peptide [96]. This suggests that such a co-encapsulation approach is not systematically needed to achieve suitable intracellular delivery of ODN.

According to the RNAi mechanism (see above), targets of ODN are located both in the cytoplasm and in the nucleus of the cells, whereas the target molecules of siRNA, i.e. mRNA, is only located in the cell cytoplasm. Thus, observations provided by confocal microscopy are useful since they allow to investigate if the nucleic acid is present or not at the subcellular targeted compartment. Since a nucleo-cytoplasmic staining of the cell was observed with transfected PACA ODNs nanocapsules [91], whereas a staining of only the cytoplasm was reported with siRNA [45], it was concluded that these nanocapsules are suitable nanocarriers for these types of RNAi agents.

Finally, the amount of nucleic acid that accumulated in the different cell compartments can be quantified by sub-cellular fractionation after gentle cell lyses. Using such a method, Toub et al. confirmed that ODN delivered with the aid of PACA nanocapsules could accumulate in both the nucleus and cytoplasm of the cells, whereas the endocytosed free ODN only accumulated in the intracellular vesicles and never reached the cell nucleus [91].

Noteworthy, the methodologies based on the measurement of the fluorescence associated with the nucleic acid cannot give proofs that the RNAi agent remained intact and biologically active. Therefore, only experiments designed to evaluate the biological activity of the encapsulated ODN and siRNA can confirm the success of the delivery method.

#### *4.1.3. Biological activity of the nucleic acid loaded nanocarriers*

In general, the methods used to evaluate the efficiency of the transfection of a siRNA or of an ODN consist in the measurement of the down-regulation of the expression of the target gene. This can be performed by quantifying the intracellular amount of both the mRNA corresponding to the target gene by Q-PCR and the protein expressed using a Western blot or an ELISA technique. In the case of a target gene coding for a fluorescent protein, the residual fluorescence resulting from the successful down-regulation of the gene caused by the siRNA or the ODN can be evaluated straight forward by FACS or spectrofluorometer. When the target gene is involved in cell proliferation phenomena (i.e. the gene c-raf [58] or the gene of the platelet-derived growth factor β-receptor PDGF βR [96]), it is also possible to evaluate the transfection efficiency by measuring cell proliferation using the MTT assay. Many parameters can influence the transfection efficiency of nucleic acids: (i) the type of nanocarrier, (ii) the nature and characteristics of the cationic polymer, (iii) the presence or the absence of serum in the cell incubation medium and (iv) the biodegradability of the chemical linkage when the nucleic acid is attached to the polymer. In the case of polymer micelles formulated with PEG-siRNA or PEG-ODN and a cationic polymer, the chemical bound has to be biodegradable to reveal the RNAi activity of the nucleic acid cargo. This was clearly demonstrated in a study comparing the transfection efficiency given by polymer micelles formulated with a non-biodegradable maleimide linked PEG-ODN and a biodegradable disulfide linked PEG-ODN. The ODN delivered by the micelles formed with the biodegradable disulfide linked PEG-ODN induced a much higher inhibition of the target gene expression than the ODN delivered by the micelles formed with the non-biodegradable PEG-ODN linkage [60].

The ester linkage used by Oishi et al. [61] was degraded in mild acid pH condition (pH 5.5) corresponding to the pH found in the endosomes, whereas no degradation was observed at pH 7.4. The degradation kinetic revealed that already one hour after the incubation of the micelle at low pH, a large part of the micelle was already degraded. In general, lysosomal accumulation after endocytosis occurs within 30–60 min. Thus, the degradation of PEG-ODN bound in the micelle was compatible with a partial release of the ODN before endo-lysosomal fusion would occur [110].

Concerning the nature and molecular weight of the polycation used in the formulation of the polymer micelles with nucleic acid bound to PEG chains, it appeared that a branched-PEI gave a superior transfection efficiency than PLL [60]. Formulations of micelles with high molecular weight polycations generally lead to high transfection efficiency but, in counter part, the toxicity of the nanocarrier was found to be increased.

Polyplexes including low molecular weight PEI (800 Da) were able to transfect cells, down-regulating by 20% the expression of the target gene [41]. A higher transfection rate could be obtained by increasing the molecular weight of PEI but an equilibrium has to be find to counterbalance the toxicity of these compounds. An interesting compromise was found by using polyplexes formulated with modified PEI of molecular weight 25 kDa. Indeed, the expression of the target gene of a siRNA associated with bisepoxydized PEI 25 kDa [42] or with ketalized PEI 25 kDa [41] was down-regulated by more than 60%. The situation appeared more confused using polyplexes formulated with chitosan. Liu et al. have shown a relationship between the transfection efficiency and the molecular weight of the chitosan used to formulate the polyplexes [107]. However, in another work, Gao et al. found that a 145 kDa chitosan gave the same transfection activity as a 21 kDa chitosan [108]. With chitosan-based polyplexes, the nature of the counterion of the chitosan ammonium group seems to play an important role in the transfection efficiency. For instance, with glutamate, a polyplex formulated with a 470 kDa chitosan showed a higher transfection activity than a polyplex formulated with the chitosan of a lower molecular weight (160 kDa). Opposite results were reported using chloride as the counterion since polyplexes formulated with a 110 kDa chitosan appeared more efficient than a polyplex including a 270 kDa chitosan [46]. The effect of the presence or of the absence of serum in the cell incubation medium has also been investigated on the transfection efficacy. No general features can, however, be drawn from these studies. According to the experiments by Urban-Klein et al. [111] serum had no influence on the transfection activity of cells by PEI – nucleic acid polyplexes. The same conclusion was drawn from a series of experiments carried out with chitosan based polyplexes, in which transfection was performed either in the presence [46,109] or in the absence of serum in the cell incubation medium [45,108]. In another experiment, Kim et al. [57] reported a loss of 60% in the down-regulation activity of the expression of the target gene when the cells were transfected with a PEI/siRNA polyplex in the presence of serum, while the activity of the siRNA was remarkable in the absence of serum. This study pointed out the necessity to shield the positive charge of the polyplex, since PEG-PEI/siRNA micelles were observed to display the same inhibition efficiency in the presence or in the absence of serum.

Noteworthy, the *in vitro* biological evaluation of siRNA or ODN conveyed by polymer nanocarriers has to be taken cautiously: a successful transfection measured *in vitro* is never a warranty for a successful delivery of nucleic acid *in vivo*. Similarly, a failure to highlight an *in vitro* activity of an intracellularly delivered ODN or siRNA does not mean that the method will be unsuccessful *in vivo*. The study published by Maksimenko et al. on the *in vitro*/*in vivo* evaluation of the transfection efficiency of an ODN targeted against a junction oncogene by means of PACA nanoplexes gives a good example of the limits of the *in vitro* assays [79]. In this study, the intracellular distribution of the fluorescent ODN clearly showed the presence of a punctuated distribution of the fluorescence inside cells indicating intracellular penetration. The measurement of the *in vitro* biological activity of the intracellular ODN failed to show any down-regulation of the targeted gene. However, these disappointed results *in vitro* did not anticipate the very successful delivery of the active ODN by means of the PACA nanoplexes *in vivo* as it was revealed on a murine experimental cancer.

### 4.2. In vivo assays

Different protocols have been suggested to evaluate the *in vivo* activity of RNAi agents delivered with the aid of polymer nanocarriers. The more straight forward protocol consists in a local injection of the formulation which avoids dilution in the body fluids and passage across biological barriers. It provides with a rapid evaluation of the efficacy of the drug delivery system, but in most cases, it remains an experimental protocol which cannot be applied to treat real disease. A more relevant protocol for clinical application includes the intravenous injection of the formulation. However, using this route of administration, the polymer nanocarrier requires a specific design of its surface properties to allow a specific recognition of the target tissue [112]. In general, for the targeting of tumors located in the organs of the mononuclear phagocyte system, nanocarriers are not needed to be surface modified. On the contrary, "stealth" nanocarriers are needed to reach tumors located outside the MPS, thanks to the enhanced permeability and retention effect (EPR). This effect, which occurs locally, results from the rapid and anarchic vascular system growth within the tumoral tissue which displays a high permeability and is accompanied by a poor lymphatic drainage [113,114]. In any cases, a further improvement in the targeting can be obtained by grafting specific ligands on the surface of the polymeric nanocarriers in order to recognize the target cells with high specificity. It has to be noted that several experiments have also considered the administration of nucleic acids associated with polymer nanocarriers by the intraperitoneal route which is another systemic route of administration [35].

Table 3 summarizes the results obtained *in vivo* with RNAi agents associated with polymer nanocarriers. Most of these studies had to do with the treatment of cancer diseases. Only a few other pathologies have been identified yet as potential candidates for RNAi therapeutic method.

#### 4.2.1. Treatment of cancer by RNAi loaded polymer nanocarriers

Treatments of tumors based on RNAi strategy can be classified in two main categories whether the target component of the siRNA or of the ODN was a product of the expression of an oncogene or whether it was linked to the intense neovascular activity found in the tumor tissue. Thus, studies performed so far were done on different experimental models of tumors using treatments based on various types of drug delivery systems loaded with either ODN or siRNA (Table 3). Although this implies that no direct comparison between the different studies can be made, the conclusions raised from these works indicated a much better performance of treatments performed with ODN or siRNA formulated in polymer nanocarriers while treatments performed with the same amount of free nucleic acids were poorly active or totally inactive. Noteworthy, interesting anticancer activity was reported even after systemic administration of RNAi agents formulated in polymer nanocarriers. In general, the superior anticancer activity given by the formulated RNAi agents could be explained by a better accumulation of the ODN or siRNA cargo in the tumoral tissue thanks to the association with the polymer nanocarrier. For instance, the tumoral growth inhibition reported after systemic injection of a siRNA/PEI polyplex in a SKOV-3 ovarian carcinoma model developed in mice was clearly correlated with an accumulation of the siRNA in the tumoral tissue [111]. Unexpectedly, such a polyplex allowed the siRNA to accumulate mainly in the tumor and in the muscle while its distribution towards the liver and the kidney was less important. Using 70 nm in diameter PEGylated micelles made of PEG-ODN/PEI, it also appeared that a high accumulation of the ODN in the tumoral tissue was a key factor for the antitumoral activity of the RNAi agent. In this study, a human lung carcinoma was implanted subcutaneously in nude mice and the ODN designed against the c-raf oncogene was administered in seven injections (one every other day) at a total dose of 17.5 mg/kg. It was found that the maximum dose of ODN reaching the tumor occurred at 12 h after injection of the micelles in the tail vein [58]. The addition of a targeting ligand to the polymer nanocarriers could further improve the efficacy of the antitumoral treatment. For example, a

**Table 3**
*In vivo* studies of RNAi based treatment vectorized with nanocarriers.

| System type | Composition | Application | Nucleic acid dose* | Reference |
|---|---|---|---|---|
| Polyplexes | jetPEI, anti-HER-2 siRNA | Human ovarian cancer<br>Intra-peritoneal | 2.4 mg/kg | [111] |
| | jetPEI, anti-PTN siRNA | Human glioblastoma | | [35] |
| | | Intra-peritoneal | 3.6 mg/kg | |
| | | Intracranial | 10 μg/kg | |
| | Chitosan 114 kDa, DD = 84%<br>Anti-GFP siRNA | Pulmonary delivery in GFP transgenic mice | 7.5 mg/kg | [45] |
| Nanoplexes | Core-shell PIHCA/chitosan<br>Anti-RhoA and anti-RhoC siRNA | Human breast cancer<br>Intravenous | 1.5 μg/kg | [81] |
| | Core-shell PIHCA/chitosan<br>Anti-EWS/Fli1 ODN | Ewing sarcoma model<br>Intratumoral injection | 1 mg/kg | [79] |
| | Core-shell PIBCA/chitosan<br>Anti-ret/PTC1 siRNA | Thyroid carcinoma model<br>Intratumoral | 1 mg/kg | [82] |
| Nanocapsules | PIBCA<br>siRNA | Ewing sarcoma model | 1 mg/kg | [92] |
| | PLGA<br>Anti-PDGFβR | Restenosis treatment<br>Local administration | 0.67 mg/kg (1 nmol) | [96] |
| | PIBCA<br>Anti-EWS/Fli1 ODN | Ewing sarcoma model<br>Intratumoral administration | 9.6 mg/kg (14.4 nmol) | [90] |
| Micelles | PEG-ODN/PEI (2 kDa, 25 kDa)<br>Anti-c-raf ODN | Human long carcinoma<br>Tail vein injection | 2.5 mg/kg | [103] |
| | PEG-siRNA/PEI (25 kDa)<br>Anti-VEGF siRNA | Murine neuroblastoma<br>Intravenous | 2 mg/kg | [36,115] |
| | PEG/cyclodextrin containing polycation/siRNA | Human Ewing sarcoma<br>Intravenous | 2.5 mg/kg | [116] |

* For the comparison, a typical mouse weight of 20 g and a molar mass of 13,400 g $mol^{-1}$ where used in the calculation.

RGD peptide (Arg-Gly-Asp) can be grafted on the PEG-PEI copolymer forming the micelles in order to target neovessels which formed in the tumors [36]. The RGD-PEG-PEI/siRNA targeted micelles were found to concentrate into the tumor very rapidly after intravenous injection (15 min) [115] and the expression of the luciferase reporter gene was clearly down-regulated inside the tumor [36]. Other ligands, like transferrin allowed the direct targeting of the tumor cells that overexpressed the corresponding receptor. Thus, micelles were formulated with polycation-adamantane/cyclodextrine complex and transferrin-PEG-adamantane/cyclodextrine complex to load a siRNA against tumor cells overexpressing the transferrin receptor [116]. Data reported by Barlet et al. [55,117] provided strong evidences that transferrin-targeted micelles were able to deliver more functional siRNA into the tumor cells than the non-targeted micelles. Indeed, the relative increase in tumor luciferase activity was 50% lower in mice treated with the transferrin-targeted micelles as compared with that of the mice treated with non-targeted micelles. Although the attachment of the transferrin targeting ligand on the surface of the micelles was found to have only a negligible impact on the extend of the accumulation of the siRNA in the tumor, it seems that the targeted system allowed a higher fraction of the siRNA to achieve intracellular localization and functional activity. Therefore, it was suggested that the targeted polymer nanocarrier contributed to enhance internalization of functional siRNA by the tumor cells [116,118].

It is interesting to point out that the way by which polymer nanocarriers are designed may influence their *in vivo* efficacy in delivering RNAi agent. For instance, the density of the targeting moieties grafted on the micelle surface was shown to influence the efficacy of the delivery system. Micelles with 1 mol% transferrin were the most effective to inhibit tumor growth on a model of mouse tumor obtained by subcutaneous injection of luciferase expressing tumor cells (Neuro2A-Luc). In comparison, the micelles grafted with 0.1 mol% transferrin and the non-targeted micelles showed less inhibition of tumor growth relative to untreated mice [118]. A low density of targeting moiety (0.1 mol%) was, however, sufficient in a metastatic xenograft model of human Ewing sarcoma, to induce an efficient tumor growth inhibition with transferrin targeted micelles [116]. According to the authors, the difference in the efficacy of the targeted micelles with different ligand densities may be caused by a variety of factors. Among those, the affinity of transferrin for the transferrin receptor found on the tumor cells is certainly not negligible. Another interesting example is given by nanoplexes which were designed to deliver ODN targeted against the EWS–Fli1 junction oncogene found in the Ewing sarcoma. The ODN-loaded nanoplexes could be obtained from two types of chitosan-coated PACA nanocarriers, one on which chitosan is simply adsorbed on the nanocarrier surface and the other, in which chitosan is chemically grafted onto the nanocarrier surface. Using a dose of ODN of 1 mg/kg, the two types of nanoplexes gave the same tumor growth inhibition after intratumoral injection. On the contrary, after intravenous administration, only the nanoplex obtained with covalently attached chitosan at the nanoparticle surface were able to produce a tumor growth inhibition effect. The nanoplexes obtained by the single adsorption of chitosan on the PACA nanoparticle surface totally loosed their antitumoral activity after intravenous administration [80]. The results obtained after intravenous administration of nanoplexes formed with the covalently attached chitosan on the surface of PACA nanoparticle were confirmed using another experimental tumor model and a siRNA as RNAi agent [81].

As mentioned above, the antitumoral activity of the delivery system may be correlated with an increase of the intracellular concentration of the functional RNAi agent. This was also confirmed by measuring the level of the expression of the targeted protein by Western blot or by evaluating the reduction of the amount of the corresponding intracellular mRNA by quantitative PCR. A very good correlation was found between the RRM2 protein knockdown and the tumoral localization of the siRNA after intratumoral administration of the siRNA associated with polymer-adamantane/cyclodextrine complex micelles in a subcutaneous Neuro2A implanted tumor [118]. Western blot analysis also confirmed the reduction of the intracellular concentration of the target protein after intravenous administration of a siRNA designed against the

EWS–Fli1 fusion oncogene formulated in transferrin targeted micelles at the dose of 2.5 mg/kg [116]. Using quantitative PCR, it was observed that the antitumoral activity given by the siRNA designed against the ret/PCT1 fusion oncogene delivered under the form of nanoplexes was accompanied by a significant reduction of the mRNA coding for the chimeric protein [82]. Besides the fact that ODN or siRNA delivered *in vivo* by means of polymer nanocarriers induced antitumoral activity thanks to a reduction of the expression of the target gene product, the high specificity of the biological activity of the RNAi agents was also demonstrated with control experiments performed with scramble or mismatch ODN or siRNA. Indeed, these nucleic acids showing an incorrect sequence were not as efficient as those bearing the specific sequence to reduce tumor growth and to diminish the expression level of the targeted mRNA and protein.

In general, the different nanocarriers used were all well tolerated by the animals. For instance, the absence of side effects was reported after intracranial injections of 15 pmol siRNA formulated as PEI/siRNA polyplexes three times per week in orthotopic glioblastoma model mice. Such a treatment was accompanied with a tumoral growth inhibition [35]. The same conclusions were drawn from the study by Hu-Lieskovan et al. [116] who have investigated the toxicity of siRNA loaded micelles and from the work of Pille [81] who stressed that the very good antitumoral effect was accompanied by a low toxicity of the treatment performed with siRNA bound to chitosan-coated PACA nanoparticles. Although the majority of the toxicity data reported so far were obtained from experiments performed in mice, one toxicity study was conducted in non-human primates after intravenous injection of transferrin targeted micelles formed using the polymer-adamantane/cyclodextrin complex systems loaded with siRNA. The experiments were performed using escalating intravenous doses of targeted micelles up to 27 mg/kg siRNA [119]. Until a dose of 9 mg/kg, the transferrin targeted micelles were well tolerated and no significant complement activation of the immune response was reported.

Besides the fact that the safety of the delivery methods has been pointed out by several authors, it is now obvious that polymer nanocarriers may efficiently protect RNAi agents from *in vivo* degradation and are able to deliver these active macromolecules in tumoral cells even after intravenous administration. So far, the main limitation concerning the application of those systems in the treatment of cancer is the delivery of siRNA or ODN from the systemic circulation into brain tumors due to the poor ability of the particulate systems to diffuse through the blood–brain barrier even if more permeable in the diseased tissue.

Another concern arises from the variety of the treatment schedules applied in the different studies which did not provided a clear view on what could be a successful protocol. Results reported from several studies suggest that tumor growth inhibition can be obtained by using rather flexible protocols. For instance, protocols in which the tumor was implanted at the same time than the treatment and those in which the treatment was given after the tumoral nodule was well established were both found relevant leading to a similar reduction of tumor growth. For example, such conclusion was drawn from a study on the delivery of ODN against Ha-ras in PACA nanoparticles coated with CTAB [120] and from another investigation in which an antiluciferase siRNA as a PEI/siRNA polyplex was used [36]. The choice of the RNAi agent, i.e. ODN or siRNA, to be used as therapeutic compound remains open as far as it includes a specific sequence. Using poly(alkylcyanoacrylate) nanocapsules as a carrier for the RNAi agent, similar control was obtained on the growth of the tumor in a model of the Ewing sarcoma implanted in mice using either phosphorothioate ODN or siRNA [90,92]. The duration of the treatment and the dose of the interfering agent were reported to influence the quality of the response. For instance, a comparison between a short- and a long-term treatment was proposed using an anti-EWS–Fli1 siRNA associated with transferrin targeted micelles to treat an experimental metastatic Ewing sarcoma obtained by subcutaneous implantation of luciferase expressing TC71 cells [116]. The short-term treatment consisted in three injections in the tail vein done at three consecutive days at a dose of 2.5 μg siRNA/kg each. An important reduction of the luciferase activity was observed during the 3 days treatment. The effect was well correlated with a reduction of the expression of the corresponding target mRNA. However, when the treatment was stopped, the luciferase activity started to increase again indicating that the tumor remained active. The long-term treatment included two injections per week over 4 weeks at the same dose than in the previous experiment. With this schedule of administration and after 3.5 weeks of treatment, no remaining visible tumor was detected using a bioluminescent imaging technique. From these experiments, it clearly appeared that treatment designed to control tumor growth might require repeated injections of the RNAi agent over a period of time of several weeks. However, this conclusion should be modulated because the response to the treatment schedule is also to depend on the tumor type. Indeed, using the same polymer nanocarrier to deliver a siRNA targeted against the replication protein RRM2 involved in the Neuro2A tumor model, Bartlett et al. have reported that the schedule of injection had no influence of on the antitumoral response. In this study, the authors concluded that a single injection of the siRNA formulation was sufficient to treat the tumor. The situation is also confused regarding the influence of the dose of the RNAi agent to be administered. Although Bartlett et al. found that there was no dose effect of siRNA administered as polymer micelles on the reduction of the tumor growth, a clear dose–response relationship was reported by Pille et al. [81] with siRNA delivered in a model of invasive MDA-MB231 breast cancer with PACA nanoparticles coated with chitosan. In this last study, a total dose of 150 μg/kg siRNA administered intravenously led to a reduction of tumor growth by 90% while a 10-fold higher dose of siRNA (1500 μg/kg) induced a clear necrosis of the tumor with a reduction of the tumor vasculature. All these data collected from the different studies provide pieces of information which are, however, sometimes contradictory. This makes difficult the task to build a kind of framework helping to design future experiments. In a very recent work, Barlet et al. [118] have attempted to build up a simulation model to offer insights for treatment design. This model was established on the basis of the extensive experience gained by the authors in investigating the delivery of siRNA with polymer nanocarriers made of micelles composed of polymer-adamantane/cyclodextrine complexes. The principle of the approach is based on the combination of data obtained from the experimental work and on theoretical consideration. To build this model, the authors suggested to include many parameters among which, a series of data concerned the tumor specificity such as its accessibility, the number of cells to be treated and the growth rate. Another set of parameters was related to the specificity of the treatment such as the threshold knockdown required, the cytostatic versus the cytotoxic character of the treatment and the duration of the therapeutic effect after the administration of a given dose.

#### *4.2.2. Other applications of therapies based on RNAi strategies*

Although antitumoral treatments are the first line of applications concerned by the RNAi strategy, other potential therapeutic applications were already identified. For instance, allergic rhinitis is another disease candidate to be treated by the down-regulation of the expression of a specific gene. Indeed, the inflammation associated with this disease is mainly due to the secretion of a cytokine, the interleukine 5, IL-5, by the T lymphocytes. Thus, by reducing the production of IL-5 by the corresponding RNAi, the inflammation should be reduced. The first type of experiments done to

investigate this hypothesis have consisted in the intranasal administration of chitosan/siRNA polyplexes to genetically modified mice expressing the green fluorescent protein in each cells [45]. The chosen siRNA sequence was targeted against the green fluorescent protein (GFP) mRNA. The level of the GFP expression was measured 6 days after a treatment performed with a single dose of the polyplex including 30 μg of the siRNA. The results showed a decrease of the fluorescence by 43% of the number of GFP positive cells compared to the untreated mice. This study showed for the first time that an efficient transfection of the cells may be obtained after the intranasal administration of siRNA/chitosan polyplexes. Such formulation was initially chosen because of the known bioadhesive properties of the chitosan. Chitosan/ODN polyplexes were then evaluated on a model of allergic rhinitis developed in mice [109]. The ODN was targeted against the IL-5 mRNA. A dose of 100 μg/mouse ODN in the polyplex was found to be able to inhibit the IL-5 secretion and subsequently to reduce the allergic reaction in ovalbumine presensitized mice. The mice treated with the polyplex showed a reduction of 58% of the intranasal IL-5 level compared to the untreated mice, whereas naked ODN could only reduce intranasal IL-5 secretion by 13%. In general, the allergic response is followed by an increase of the level of the IgE; such increase was not observed after the treatment with the polyplexes. A further confirmation was given by the histological study of the nasal mucus which revealed a decrease of the inflammatory response due to the allergen.

Another interesting application is the control of restenosis after transluminal coronary angioplasty. In this indication, the aim of the treatment is to control the proliferation and the migration of smooth muscle cells from the media layer to the intima which is the main cause of restenosis. Hence, the delivery to the smooth muscle cells of a RNAi agent targeted against the growth factor receptor involved in the restenosis mechanism was expected to inhibit the neointimal formation [96]. In this view, PLGA nanocapsules were loaded with an ODN targeted against the platelet-derived growth factor beta-receptor (PDGFβR). The performance of the treatment was evaluated 14 days after local injection in a balloon-injured rat restenosis model. The free ODN showed a significant antirestenosis effect by itself but it was at the same level as the effect observed after treatment with empty nanocapsules. On the contrary, the antirestenosis effect reported after the treatment with the ODN-loaded nanocapsules was much higher, indicating that the PLGA nanocapsules could serve as an effective delivery system of RNAi to control restenosis after coronary angioplasty.

## 5. Conclusion

Efficient *in vivo* delivery of RNAi effectors is the key of success for the development of new highly specific therapeutic methods based on RNA interfering approaches. Progresses in polymer chemistry and in the design of adapted polymer nanocarriers have provided efficient formulations to deliver RNAi agents *in vivo*. Proofs have been given that such systems can protect ODN or siRNA from degradation by nucleases even when they are in the *in vivo* environment. Clear evidences are given by the literature that polymer nanocarriers can, indeed, be used to deliver functional ODN or siRNA to their target cells after intravenous administration. Antitumor treatments based on this strategy were found successful in different experimental tumor models. As suggested by the recent reports, the application of RNAi nanocarriers is not restricted to cancer but can be extended to other diseases at the condition that the pathological process can be corrected by the down-regulation of the expression of a known gene.

Regarding the safety, no redhibitory toxic reaction or side effects have been reported so far after the administration of RNAi polymer nanocarriers. Although the transfection efficiency of these artificial nanocarriers is generally lower than with viral vectors, they appear much safer for future applications in humans. Thus, it is expected that the polymer nanocarriers will continue to be developed in the future as a platform for the *in vivo* delivery of RNAi agents. However, systems formulated without polycationic compounds may have some advantages to better suit the compliance imposed by health authorities and to reach clinical development stages.

In our view, this field of research will most probably extend by the development of nanodevices decorated with specific ligands which will further improve both the specificity of the delivery to the target cells and the transfection efficiency. For this purpose, structured nanocarriers like nanoplexes or polymer micelles are suited with the fact that the targeting ligand will be correctly oriented at the surface of the nanocarrier. On the contrary, disordered structure of polyplexes impairs the orientation. Although very similar to natural virus, the binding of targeting ligand onto the nanocaspule surface remains a big challenge because of their fragility. More efforts will certainly concentrate in the near future on the development of polymer nanocarriers with better controllable drug releasing properties which will bring more nucleic acid in the targeted cells and intracellular compartments. Together, the earlier reports based on the delivery of RNAi agents by means of polymer nanocarriers raise considerable hope to bring into clinics novel therapeutic RNAi-based concepts in human.

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