Chitosan and its derivatives as nanocarriers for siRNA delivery

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List of abbreviations:

Ago2: argonaute 2; AIDS: acquired immune deficiency syndrome; ATPase: adenosine triphosphatase; dsRNA: double stranded ribonucleic acid; DNA: Deoxyribonucleic acid; DD: deacetylation degree; EGFP: enhanced green fluorescent protein; GC: glycol-chitosan; GFP: green fluorescent protein; HER2: human epidermal growth factor receptor 2; HMWC: high molecular weight chitosan; HTF: Tenon's capsule fibroblasts; IKKβ: IκB kinase beta; kDa: kilo Dalton; LMWC: low molecular weight chitosan; LNA: locked nucleic acid; MAA: methacrylic acid copolymer; MR: magnetic resonance; mRNA: messenger ribonucleic acid; miRNA: micro

ribonucleic acid; mPEG: polyethylene glycol monomethyl ether; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Mw: molecular weight; NF-κB: nuclear factor kappa B; PCI: photochemical internalization; pDNA: plasmid deoxyribonucleic acid; PEI: polyethyleneimine; PLGA: poly(lactic-co-glycolic) acid; PIBCA: poly (isobutylcyanoacrylate); PIHCA: poly (isohexylcyano acrylate); PLR: Poly-L-arginine; QD: quantum dots; RGD: Arg-Gly-Asp peptide; RISC: ribonucleic acid-induced silencing complex; RLN: relaxin; RNA: ribonucleic acid; RNase III: ribonuclease III; RNAi: ribonucleic acid interference; shRNA: short hairpin ribonucleic acid; siRNA: small interfering ribonucleic acid; RFP: red fluorescent protein; RSV: respiratory syncytial virus; TMC: trimethylchitosan; TNF-α: tumor necrosis factor; TPP: tripolyphosphate; UTR: untranslated region; VEGF-A: vascular endothelial growth factor-A

Abstract

The ability to specifically silence genes using siRNA has enormous potential for treating genetic diseases. However, siRNA instability and biodistribution issues still need to be overcome, and adequate delivery vehicles have proven indispensable in conveying siRNA to its target. Chitosan is a promising biopolymer for siRNA delivery, its interest stemming from its safety, biodegradability, mucoadhesivity, permeation enhancing effect and cationic charge, as well as amenability to undergo chemical modifications. Chitosan and its derivatives can be readily arranged into complexes or nanoparticles able to entrap and carry siRNA. Specific strategies have been adopted to improve chitosan-based vectors with regard to transfectability. However, further efforts are required to verify their value and adapt them to enhance therapeutic output prior to clinical application. This review emphasizes the potential of chitosan and its derivatives to develop nanocarriers for siRNA delivery. The properties of chitosan that are significant for transfectability and the most relevant findings are assessed.

Key words: Chitosan, Chitosan derivatives, Formulation parameters, Gene silencing, Nanocarriers, siRNA Delivery

1. Introduction

Recently, the world has witnessed an explosion of knowledge concerning RNA and DNA-based diverse functionalities in molecular biology and their potential in therapeutics applications. RNA interference (RNAi) therapeutics represents nowadays a potential new class of pharmaceutical drugs, arising from the fact that they are easy to design and specific for targeting [1-3].

Essentially, RNAi is a naturally occurring phenomenon where endogenously processed RNA molecules mediate sequence-specific gene regulation. It was first described for the nematode worm *Caenorhabditis elegans* by the Nobel Prizes *Craig Mello* and *Andrew Fire in* 1998 [3]. This

mechanism is triggered by antisense molecules like oligonucleotides, aptamers, shRNA (short hairpin RNA), microRNA, siRNA (small interfering double-stranded RNA) and ribozymes [2,4], that target a complementary messenger RNA (mRNA). In early 2000, siRNA was interestingly shown to mediate sequence-specific translational block of the target mRNA in mammalian cells *in vitro;* and, was successfully delivered to mice. Fascinatingly, some of siRNA-based therapeutics is actually in clinical trials, mostly directed to ocular treatment. The first clinical assessment of siRNA-based on human therapeutics occurred in 2004 and was directed at the treatment of wet age-related macular degeneration, whereas Vitravene® is the first siRNA-based product available on the market for the treatment of cytomegalovirus-induced retinitis in AIDS patients. The first systemic siRNA administration of siRNA in non-human primates was reported in 2007 [4-7].

The antisense function of siRNA can be exogenously induced by the introduction of chemically (preformed) or enzymatically (expressed intracellularly via DNA vector) synthesized siRNAs, yielding transient or more durable knockdown, respectively [6]. These simulate the RNAi machinery in eukaryotics where suppression of target genes is achieved through excising long double-stranded RNAs into siRNAs, by a cytoplasmic ribonuclease action called dsRNA-specific RNaseIII enzyme dicer. This mechanism results in a transient and reversible RNAi effect. Also, intracellular generation of siRNA could be endogenously induced via plasmid or virus-driven precursor small hairpin RNAs, named natural dsRNA-encoding genes or microRNA genes (miRNAs). They result in long lasting effects (weeks or months) and, thereby, could be useful for chronic diseases such as cancer [6]. Moreover, they require multiple enzymatic steps prior to targeting mRNA degradation (transcription, nuclear export and dicer processing) [9]. In either case, the double-stranded RNAs (dsRNAs) of 21-23 nucleotides associate in the cytoplasm with a protein complex called RNA-induced silencing complex (RISC); the multiple-turnover enzyme complex that mediates endonucleolytic cleavage in the RNAi pathway. siRNA is loaded onto Argonaute 2 (Ago2), which is the core catalytic component of RISC required for the unwinding of siRNA duplex

and, consequently, assembling of siRNA into RISC. RISC subsequently cleaves and releases one strand of siRNA (passenger strand), resulting in an activated form of RISC with the other single-strand (guide siRNA) that directs the specificity of the targeted mRNA recognition through complementary base pairing. Another enzyme of RISC, called endonuclease, cleaves the phosphodiester bond on the mRNA in the middle of the siRNA-mRNA recognition site, releasing the cleaved mRNA fragments (between bases 10 and 11 relative to the 5′ end of the siRNA antisense strand) [10-13].This step leads to mRNA degradation (in the case of siRNAs) and/or translational repression by binding to the 3′ untranslated region (UTR) (in the case of miRNAs) and, eventually gene silencing [6,8,9,14]. The siRNA loaded RISC can be recycled for multiple rounds of silencing; a feature that makes siRNA more tempting for therapeutic purposes because very small siRNA doses are required to achieve the desired effect. This also results in reduced costs and possible concentration-dependent off-target effects. It is worth noting that the ability of siRNA to form a hybrid with the targeted mRNA depends on its sequence specificity, as well as its affinity, which is determined by the number of H bonding formed between siRNA and the targeted sequence [12].

Compared to other antisense molecules, siRNA comprises of a larger portion of the RNA interference pathway that induces sequence-specific inhibition of gene expression, showing perfect complementarity to the target mRNA. It is also regarded as potent since only a few molecules of siRNA per cell are required to produce the antisense effect [15]. Apart from its low molecular weight (Mw), which is 100 times lower than, for example, shRNA, it acts in the cytosol rather than in the nucleus, which implies facing fewer obstacles during delivery [13]. Besides, siRNAs are easy to design and selectively specific for targeting [16-18]. In light of these findings, siRNA has proved to be more efficient in gene silencing than other RNAi molecules and opened wide perspectives in therapeutics for the treatment of any disease that is linked to elevated expression of an identified gene, such as cancer, infectious, inflammatory and neurodegenerative diseases. However, the most

challenging issue in the siRNA-based therapy is its delivery to the target site, particularly due to the lack of stability of siRNA in physiological fluids and the poor penetration into cells. As it is known, gene delivery is, generally, a multistep process, thus facing many extra- and intracellular difficulties concerning the transport of therapeutic nucleic acids to the targets; a consequence which has spurred research to seek out novel delivery strategies. In addition to the hurdles related to the siRNA nature, other challenges may depend on both the target organ and the route of administration, this being less pronounced in the case of locoregional delivery [13]. Excitingly, there have been many innovative technologies focusing on solving this issue which have met the necessary requirements related to the carriers used, the route of administration and the specific target [9].

The delivery systems proposed so far include viral [19] and non-viral vectors, the latter being based on nucleic acids [20] or encapsulation within a carrier system. The approach of non-viral carrier design requires the use of materials such as cationic lipids [21], polymers [22], dendrimers [23], proteins and peptides [24] as well as metallic nanoconstructs [25] and polyamino acids [26], mainly in the form of nanoparticulate systems [11,12,27]. Interestingly, matrix-based siRNA delivery using tissue engineering scaffolds has been also explored, taking advantage of sustained release and high payload protection conferred by such devices [28]. Noteworthy, an effective delivery system must exhibit low toxicity, high siRNA encapsulation efficiency, siRNA stabilization during either processing or delivery, long circulation time, cell-specific recognition, capacity for cellular internalization, endosomal release and cytoplasmic localization with efficient dissociation prior to acting on the target [6,8,9,29-31]. Thus, the carrier may enable precise control over the onset and duration of antisense action, resulting in a high and more long-lasting therapeutic output. In addition to these chemical-based methods, siRNA delivery has also been described using physical methods, such as hydrodynamic delivery, which is still restricted to animal application [32], and electroporation [33]. Due to concerns associated with the application of viral vectors,

including mutagenesis, immunogenicity and pathogenesis [34], together with the recognized toxicity and low physical stability of cationic lipids, siRNA delivery strategies lean towards the development of polymeric vectors, considering their ease of preparation, compared to the aforementioned vectors, and the possibility of conceiving multifunctional delivery to substantiate siRNA therapy *in vivo*. In this context, chitosan is positioned as outstanding candidate given its interesting properties for siRNA packaging or encapsulation and cellular uptake, as demonstrated by the extensive researches investigating this polymer for siRNA delivery purposes [30,35].

This review article highlights the interest of the cationic polymer chitosan as a potential nanovector for siRNA delivery. We present an overview of the efforts done to potentiate the role of chitosan in gene silencing through addressing parameters related to its formulation and structure, namely derivatization, elucidating the consequent effects both *in vitro* and *in vivo*. In addition, insights into structure-activity relationship and the state-of- the art of siRNA delivery systems based on chitosan will be provided.

2. Role of nanotechnology in siRNA delivery

As stated above, siRNA delivery is confronted with several hurdles related to either the siRNA nature or the physiological barriers. Moreover, the physicochemical properties of siRNA, such as hydrophilicity, high molecular weight and negative charge [11-13], render poor cell penetration followed by low transfectivity when administered as a naked molecule [36-38]. Nanotechnology tools have been beneficial in circumventing these obstacles, which can be summarized as follows:

a) *Potential siRNA toxicity*: in spite of its reported safety, due to the metabolism of natural nucleotides in cells, siRNA potential toxicity may arise from the induction of innate immunity, off-target gene silencing and competition with endogenous RNAi components (e.g. miRNAs) for limited RNAi pathways. This potential toxicity originates from some immunostimulatory motifs

during siRNA design, and can be fortunately solved by chemical modification which results in a higher therapeutic effect at smaller doses and also provides selectivity, evading interference with other RNAi molecules [6,13,36].

b) *Extracellular barriers:* it is worth mentioning that most tissues are not easily accessible and require the development of effective delivery vehicles. The implement of nanotechnology, to design siRNA nanocarriers, has become appealing in order to overcome these barriers which hamper transport and stability and, consequently ensure sufficient siRNA delivery with maintained integrity. Extracellular barriers, if systemic administration is intended, encompass interactions with body enzymes like nucleases and proteins as well as removal by glomerular filtration [13]. Furthermore, the rapid elimination and, hence, short half-lives (around 5 min), of exogenous siRNA, require repeated administration to attain a determined therapeutic effect [6,37].

c) *Targetability*: cell-type specific delivery has been tackled using targeting moieties or ligands either tagged directly to siRNA or to the siRNA carrier [10,39]. These may include antibodies [40,41], ligands [42,43], aptamers of other DNA/RNA hybrids [44] and peptides [45].

d) *Cellular uptake and intracellular trafficking:* challenging barriers impair siRNA delivery to the target tissue. Cellular uptake and intracellular trafficking constitute complex steps which require specific delivery considerations. In fact, siRNA is internalized into cells via endocytosis through endocytotic vesicles that fuse with the early endosomes [2,10,13]. These subsequently mature into late endosomes prior to fusing with lysosomes, whose luminal pH is mildly acidic (4.5-6), thus leading to siRNA degradation and, hence, low bioactivity. Therefore, siRNA must be able to escape early from the endosomes, to avoid ending up in the lysosomes. Nonetheless, siRNA cellular uptake may be achieved without using the endosomal pathway, which has been postulated to occur with, for example, cell penetrating peptides [46] and cholesterol [42].

For lysosomal escape of siRNA, membrane destabilizing agents have been used to promote its release into the cytosol in a sufficient quantity, such as fusogenic peptides [47] and lipids [48], as

well as pH-responsive lipids [49] or polymers [18] and reduction-sensitive polymers [50,51]. The pH-responsive polymers, containing protonable amines, such as the cationic ones, are hypothesized to have a proton sponge effect or buffering capacity whereby proton influx is initiated through a vesicular ATPase-driven pump. Then, electrostatic repulsion occurs between the protonated amine groups leading to complex swelling or expansion, which results in raising internal osmotic pressure and counter-ion penetration to end up with endosomal membrane rupture and, subsequently, siRNA release [13,18]. Endosomal escape might also be mediated by reduction, generated through a disulfide bond [2].

As referred to above, nanotechnology has been providing solutions for bypassing the limitations of siRNA delivery, in order to enhance its therapeutic output, by engineering delivery approaches particularly endowed with multifunctionality. These strategies may include the direct modification of siRNA molecules, which may involve either tailoring the siRNA length [9,14] and/or the chemical modification of the siRNA sense strand. This has resulted in enhanced serum stability with an increasing half-time *in vivo*, reduced sequence-dependent off-target effect and immunogenicity and, consequently, improved silencing activity [2,8,37]. On the other hand, in order to produce a biologically active siRNA, the selection of a proper siRNA sequence is the pivotal step in the synthetic siRNA design as this selection mainly depends on that of the targeted mRNA. After identifying the sequence to perform the effect of interest, further structural and chemical modification for siRNA are still needed to enhance its biological performance *in vivo* [37]. Chemical modification can be achieved by replacing the phosphate backbone through phosphorothionate or boranophosphonate linkages, or the 2-hydrocyl group of the ribose sugar with 2′-amine, 2′-halogen, 2′-O-methyl and locked nucleic acid. In addition, these modifications may also be performed on the nucleobase and on the 5′- or 3′- terminus, where end modification of siRNA allows conjugation with ligands, antibodies and other targeting moieties [8,9,10]. In this

context, it has been found that the sense strand and the terminal regions of siRNA can tolerate more modifications than its antisense strand and the central region [8].

Notwithstanding the possibility of siRNA modification, the molecule is still subject to renal filtration when administered to the blood stream, owing to its small size (< 10 nm). Furthermore, a poor pharmacokinetic profile is observed due to its polyanionic character, and its conjugation often makes endosomal release a difficult task, apart from impairing affinity to the target [10]. Thereby, delivery strategies have been pursued to generate new potent vectors, such as the already mentioned polymeric vehicles.

3. Interest of chitosan in siRNA delivery systems

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Figure 1) that is derived from chitin, and is one of the most promising biopolymers for drug and gene delivery. It has been largely investigated owing to its interesting and broad biological activities, which include wound healing [52], anti-tumor properties [53], as well as hypocholesterolemic [54], antimicrobial [55] and antioxidative [56] effect. It has also been extensively studied for drug delivery purposes, demonstrating low toxicity, biodegradability and biocompatibility [57-59], as well as mucoadhesivity [60,61] and the enhancement of macromolecules permeation through triggering the opening of epithelial tight junctions [62]. The chitosan structure is amenable to chemical derivatization; therefore its physicochemical properties can be modulated to fit specific requirements that ensure efficient delivery [63]. Chitosan displays a cationic character due to the presence of amine groups. This characteristic is essential for siRNA packaging or encapsulation which, in turn, provides protection against degradation during either processing or delivery and facilitates its cellular uptake, as well as the endosomal escape, due to the buffering capacity of this

polymer [30]. It is worth mentioning that chitosan was first employed in gene therapy in 1995 by *Mumper et al* [64].

The most popular techniques to prepare chitosan-based siRNA delivery systems comprise of polyelectrolyte complexation [38,65], coacervation [17,66] and ionotropic gelation [67,68]. In these techniques, electrostatic interactions are involved whereby the positively charged amine groups of chitosan interact with the negatively charged phosphate groups of siRNA in addition to the crosslinker tripolyphosphate (TPP), in the case of gelation technique [30,63]. During condensation reactions, the extended siRNA is transformed into the condensed state, driven by the overall increase in the entropy of the system, leading to the counter-ions release which consequently results in compaction [69]. The resultant structures are of defined morphology and reduced dimensions, thus their cellular uptake is expedited via endocytosis, by shielding the negative charge of siRNA that hampers its interaction with biological membranes (typical molecular weight of siRNA is approx. 15 kDa) [70,71]. Desolvation has hardly ever been used in this regard [30,72]. Collectively, these techniques result in cationic siRNA vehicles, which benefit the interactions with biological membranes, as previously mentioned [72,73]. Chitosan-based gene vectors were initially designed as polyplexes, resulting from a simple complexation between chitosan and genetic material. After that, because of physical and biological problems affecting their ability in gene transfection, *Köping-Höggård et al*. adopted the ionic gelation technique, previously developed for protein encapsulation, in order to alternatively incorporate genetic material into nanoparticulate systems [74]. siRNA delivery in the form of chitosan nanoparticles was described to enhance cellular uptake by up to 1.8-fold compared to the chitosan solution in A459 cells, without notable influence on the chitosan toxicity profile [75]. Nevertheless, in a work with murine fibroblasts, *Dehousse et al*. found that higher cell toxicity was observed upon incubation with a solution of trimethylchitosan, compared to the nanoparticulate form, probably because the cationic charge is reduced upon complexation and nanoparticle formation, decreasing the interaction with cell membrane [27].

Furthermore, in contrast with the results obtained for the chitosan solution, chitosan nanoparticles uptake has shown to be a saturable event, with the binding affinity and uptake capacity decreasing with the reduction of polymer molecular weight and degree of deacetylation [76]. The internalization of chitosan nanoparticles appeared to predominantly occur by adsorptive endocytosis, initiated by non-specific interactions between nanoparticles and cell membranes, and was in part mediated by the clathrin-mediated process [75]. Chitosan has been demonstrated to have potential in modulating siRNA stability and biodistribution [37].

In order to investigate the optimal conditions for satisfactory levels of gene transfection, it is necessary to study the physicochemical characteristics of chitosan-based siRNA systems through the variation of particular parameters and correlate them with transfection efficiency. These parameters include the preparation conditions (pH and ionic strength) and mass or N/P ratio (ratio of chitosan amino groups to siRNA phosphate groups) [26,67,77] as well as the structural properties of chitosan, such as molecular weight (Mw), degree of deacetylation (DD), chitosan type [77,79] and chemical modification [27], which affect the specific interaction with siRNA enormously. The role of these parameters in transfection efficiency is visible in their direct effect on physicochemical properties of chitosan particles that eventually control siRNA delivery kinetics *in vivo*, including cellular interactions [38]. These particle properties encompass particle size, zeta potential and stability, as well as the association efficiency of siRNA and binding capacity. Below, a thorough description of the various factors referred to which affect siRNA delivery is provided.

3.1. *Effect of chitosan molecular weight*

Chitosan Mw has been demonstrated to greatly influence transfection efficiency, as it translates to a direct effect on particle size, stability and, consequently, on the siRNA release behavior [80]. *Techaarpornkul et al.* reported that complexes prepared at a chitosan/siRNA weight ratio of 32, based on low molecular weight chitosan (LMWC) (20 kDa, DD = 85%) manifested a

higher enhanced green fluorescent protein (EGFP) silencing effect than those based on high molecular weight chitosan (HMWC) (200 and 460 kDa) in EGFP stably expressing human cervix epithelial carcinoma (Hela) cells. In this case, the effective gene knockdown was attributed to the smallest sizes of particles based on LMWC, although HMWC resulted in more stable complexes. The higher stability observed for complexes of HMWC could be explained by a chain entanglement effect, whereby chitosan easily entangles free siRNA, resulting in stronger binding to siRNA and, thus increased stability, but reduced siRNA decoupling in the cytosol prior to acting on its target mRNA. This might rationalize the lower transfectability of the complexes based on HMWC [79]. Another work by the group has drawn the same conclusion in which the effect of chitosan Mw on gene silencing activity was also studied. However, on the contrary of what was described in the first study, particle size was shown to be dependent on chitosan Mw, between 20 and 200 kDa [16]. The proportional correlation between particle size and Mw has been documented in several other works [67,81], although in some cases smaller particle sizes were reported with increasing Mw of chitosan (from 140 to 250 kDa), possibly due to increased inter-chain connections between longer chitosan molecules [82]. Surprisingly, in this latter study, this difference in particle size did not significantly affect transfection efficiency; also implying that Mw did not induce differences in the transfection level. Nevertheless, transfection was more effective than that of the commercial Lipofectamine® control [82]. Likewise, other authors report that the transfectability of complexes composed of chitosan or trimethyl chitosan/siRNA in HEK293 cells was independent of chitosan Mw (42-400 kDa, DD = 84-88%) [27]. This may suggest that chitosan Mw will not remarkably affect the transfection efficiency by itself. Continuing in the same context, *Liu et al.* [80] studied differences in transfection efficiency resulting from the application of chitosan with variable Mw and DD in EGFP expressing H1299 cells (human lung carcinoma cells). In contrast to the aforementioned observations, higher gene silencing was found for HMWC, being concomitantly dependent on DD, with better transfection results for high DD. To establish this conclusion, chitosan with Mw of 8.9-

173 kDa was used, and the resulting complexes featured smaller sizes and improved stability at higher Mws, which was attributed to the availability of a sufficient chain length and charge density to condense siRNA into discrete particles. This indicates that the presence of chitosan chains which are long enough, along with H-bonding and the hydrophobic interactions between the sugar residues of chitosan as well as the nucleoside bases of siRNA, may further contribute to the mechanism of complexes formation. Similarly, HMWC of 140 kDa (DD = 84%) could efficiently knockdown EGFP gene in H1299 cells at 50 nM of EGFP-siRNA, thus being comparable with the *Trans*IT-TKO siRNA transfection agent. It also protected siRNA against nuclease breakdown, maintaining it intact after incubation in serum [65]. Besides, chitosan-glutamate of 470 kDa (DD = 86%) demonstrated significant gene silencing effect following transfection of CHO K1 or HEK 293 cells with chitosan/TPP/siRNA nanoparticles (82%), compared to the LMWC of 160 kDa. Moreover, the observed effect was comparable with that of Lipofectamine® 2000 [67].

Although in some cases results reported in different studies are not coincident, and on occasions are even contradictory, which is probably a result of different assay conditions in general, it is worth noting that chitosan with Mw below 10 kDa failed to form siRNA complexes, which can be attributed to shorter and stiffer chitosan chains that restrict inter-winding [80]. This contradicts what has been observed for pDNA, where LMWC is generally recognized to mediate higher gene transfection [83-85].

3.2. *Effect of chitosan deacetylation degree*

The contribution of DD to particle formation and, consequently, to the resultant gene silencing effect is related to the steric hindrance provided by chitosan molecules, where the bulky acetyl groups (lower DD) minimize siRNA binding to chitosan. As a result, particle stability decreases, leading to premature siRNA release and even lower protection against serum proteins [86]. This lower DD, which confers less charge intensity, further explains the formation of less

compact and spherical structures, by virtue of weaker ionic interactions between polyelectrolytes [27]. Despite the higher cell viability observed at lower DD, reduced epithelial penetration mediated by the opening of tight junctions in Caco-2 cells was also found [87]. In addition, high DD results in highly stable particles and, consequently lower siRNA disassembly. These consequences impair efficient gene transfection. Taking into account that chitosan degradation kinetics increase with the extent of acetylation, therefore, an adequate DD is required to achieve a balance between the degradation rate and stability of chitosan complexes as well as to provide a more controlled release of siRNA [2,29,30,87]. *Liu et al*. studied the effect of chitosan DD (54-95%) on gene transfection efficiency. It was found that siRNA complexes formulated with chitosan of higher DD exhibited higher gene transfection, whereas those prepared with chitosan of the same Mw but lower DD, exhibited trivial gene regulation [80].

More than affecting the gene silencing effect, DD has been shown to influence cellular uptake. Interestingly, at a specific DD (70% deacetylated chitin), chitosan has also been shown to provide adjuvant properties for macrophage stimulation [88].

3.3. *Effect of chitosan type*

Techaarpornkul et al. reported that siRNA complexes produced with chitosan hydrochloride and chitosan acetate exhibited slightly better stability compared to those made of chitosan glutamate and aspartate. The chitosan salt form affected both complex size and zeta potential. Nonetheless, at a specific weight ratio, it had only a trivial effect on transfection efficiency. Complexes based on different salts (hydrochloride, glutamate, acetate, aspartate) showed a comparative level of gene knockdown, which was higher than that of the chitosan acetate control [79]. *Katas et al*. found that chitosan-glutamate induced considerably higher transfection levels after siRNA delivery into both HEK 293 and CHO K1 cells, compared to chitosan-hydrochloride. This could be attributed to the smaller size of the nanoparticles made of chitosan-glutamate, although it had larger Mw [67].

3.4. *Effect of mass or N/P ratio*

Contrary to what has been experienced with chitosan/pDNA complexes, some studies have shown that high mass or N/P ratios of chitosan/siRNA are needed in order to obtain complete nanocomplexes displaying low zeta potentials. These findings strongly indicate that the siRNA mechanism of binding to chitosan is distinct from that of pDNA. This is reflected in the easier pDNA binding to chitosan compared to that of siRNA, which could be explained by the difference in size and structure between both molecules. It is postulated that pDNA is easily wrapped up with chitosan chains, whereas siRNA is short and has a rigid structure, which protrudes out of the chitosan chains and, thus results in less positive net surface charge [17,71].

Furthermore, high mass or N/P ratios contributed to increment the positive charge, which led to more stable complexes with more compact structures (smaller sizes) [16,81,89]. This property substantiated and prolonged transfection effect; a consequence that could be attributed to the higher number of complete complexes entering cells, followed by higher siRNA amount released in the cytosol. Furthermore, the higher stability of these complexes, owing to stronger binding to siRNA achieved at higher ratios, may also clarify this effect. Conversely, complexes at lower ratios display a faster release due to less binding strength. In addition, the more efficient transfection, for chitosan/siRNA complexes at higher ratios, could be a consequence of higher cell internalization that precedes the antisense activity. For instance, *Liao et al*. noticed more prominent internalization of chitosan/siRNA complexes at higher ratios in human fibrosarcoma cells [26]. *Techaarpornkul et al*. also found substantially higher transfection for complexes at the mass ratio of 32 compared to those at 4, in EGFP-expressing Hela cells [79]. Similarly, *Liu et al.* found a greater knockdown of the same gene at N/P of 50 and 150 than that of 2 and 10 in EGFP-expressing H1299 cells [80]. Others have also reported the same conclusion with different chitosan formulations and cell lines, as well as *in vivo* [16,22,26,89]. However, greater ratios have been described to induce cell toxicity due

to increased positive charge of the carrier or high concentration of the polymer [26,65,90]. Hence, optimal mass or N/P ratio should not only refer to transfection efficiency, but also to a satisfactory toxicity feature. On the other hand, increasing chitosan concentration relative to that of siRNA implies increased mass or N/P ratio, which has been demonstrated to be accompanied by a decrease in particle size, probably the increased polymer amount leads to tighter complexes [16,81,89-91]. In other words, increasing siRNA concentration decreases this ratio, resulting in increased particle size which may be due to increased siRNA bond-forming bridges between chitosan chains, consequently leading to greater chitosan incorporation [65]. On the contrary, *Katas et al*. observed size increment for nanoparticles at higher chitosan concentrations or chitosan/siRNA weight ratios, using different Mw and types of chitosan. Moreover, nanoparticles exhibited higher physical stability when chitosan/siRNA weight ratio approached 100:1 [67]. Also, *Lee et al*., noticed the same tendency where particle size increased along with increased weight ratio of siRNA nanoparticles, composed of chitosan (470 kDa, DD = 86%) and polyguluronate [17]. However, these studies did not address the effect of the obtained weight ratios on transfection efficacy.

According to all that has been commented on, it is suggested that the mass or N/P ratio greatly influences gene transfection through controlling particles' size, surface charge and binding strength. Nonetheless, this ratio must be appropriately optimized in order to accomplish an adequate transfection level without deleterious toxic effects.

3.5. *Effect of pH*

The pH of both siRNA formulation and culture medium has also been described to affect transfection kinetics by controlling the binding events between siRNA and chitosan, which determine the complex stability and, therefore, siRNA release rate. For instance, at a lower pH, chitosan presents a higher charge density, due to amine protonation, extendedly favoring the flexible-like structure, owing to the repulsion between its protonated amines. This chitosan

conformation allows more binding contacts with siRNA, resulting in stronger interactions and leading to globular, compact and stable complexes. In contrast, at a higher pH**,** deprotonation prevails and chitosan conformation changes due to the collapse of electrostatic interactions, resulting in weaker binding to siRNA, with consequent faster siRNA release [77].

Mittnacht et al. reported that the pH had no effect on particles size [82]. Contrastingly, other studies demonstrated that a pH modification markedly influenced the size, zeta potential and physical stability of chitosan or trimethyl chitosan/siRNA nanoparticles [27,81]. A study by *Laroui et al.* evidenced the dependency of the size of chitosan/siRNA complexes on the pH as well [92]. On the other hand, *Katas and Alpar* observed that the particles' surface charge increased 2-fold to +40 mV when the pH of chitosan solution changed from 6 to 4, without observing a significant difference on particles size in this range of pH [67]. Nevertheless, the nanoparticles obtained at pH 4.5 were the smallest in size, which supports the idea that chitosan amines are mostly protonated, hence binding more tightly to siRNA and resulting in smaller sizes. Taking into account that pKa of chitosan is around 6.5, *Rojanarata et al.* showed that the inhibition of gene expression was dramatically decreased for chitosan-lactate/siRNA complexes when the pH of culture medium increased up to 7.2. This effect was mitigated by complexing the siRNA with a chitosan derivative, which resulted in a more stable system that induced higher gene inhibition [16].

4. Nanocarrier-based siRNA delivery using native chitosan

Chitosan has been directly complexed with siRNA to prepare suitable formulations for gene transfection. For instance, *Liu et al.* transfected enhanced green fluorescent protein (EGFP)-expressing H1299 human lung carcinoma cells with chitosan/siRNA (chitosan Mw = 114 and 170 kDa, chitosan DD = 84%; N/P = 50 and 150) complexes, which resulted in an efficient gene knockdown (80%), comparable to that of the *Trans*IT-TKO® siRNA Transfection Reagent [80]. Using the same cell line and chitosan with the same molecular weight (114 kDa, 84%), the above

group reported thereafter similar results in the presence of serum and with 50 nM of EGFP-siRNA (77.9%, N/P = 57), at 48 hours post-transfection. Similar results were also attained in primary cells harvested from EGFP-transgenic mice (86.9%, N/P = 36), thus being the first study on transfecting primary cells with a polycation-based system (N/P = 57). This also resulted in 90% knockdown of BCR/ABL-1 oncogen protein, following transfection with the therapeutic BCR/ABL-1 siRNA in K562 cells (human myelogenous leukemia line) [65]. These complexes (350-450 nm, N/P = 36) further down regulated tumor necrosis factor (TNF-α) in systemic and peritoneal macrophages and, consequently, arrested joint swelling in collagen-induced arthritic mice, following intraperitoneal injection. Besides, the use of 2′-O-Me-modified siRNA did not induce the innate immunity in macrophages, compared to its unmodified counterpart [93].

It is worth noting here that they also modified the EGFP-siRNA with locked nucleic acid (LNA) to increase its stability. Similar to the previous experiment, the same chitosan complexes, containing the modified siLNA, were intranasally administered (30 μg siRNA/day for 5 days, N/P = 7). Transfection with these complexes led to 50% knockdown of EGFP in lung mice epithelium compared to siLNA-mismatch control. This silencing outcome was also higher than that of the unmodified siRNA, as revealed in the previous study (37%). The naked siLNA did not induce knockdown which is presumably due to the mucoadhesive properties of chitosan. The results obtained in this study support the idea that, for optimal siRNA delivery, chemical modification of siRNA may be required in addition to a suitable carrier system [36].

With a view to developing inhalable RNAi-based therapeutics, this system was further investigated for pulmonary RNAi delivery following intranasal administration in EGFP-transgenic mice (Mw = 114 kDa, DD = 84%, N/P = 6). Results showed that 43 and 37% knockdown in EGFP-expressing bronchiole epithelial cells compared to untreated mice and EGFP-mismatch control, respectively [65]. Following that, local lung delivery using a non-invasive intratracheal insertion of a nebulizing catheter, was implemented into mice (chitosan Mw = 170 kDa, DD = 84%) [94]. The

evaluation of aerosol lung deposition and gene silencing in H1299 cells (N/P = 57) revealed that aerosolization altered neither particle size nor silencing efficiency of the tested formulation at 50 nM of siRNA, which further showed minimal effect on cell viability compared to the *Trans*IT-TKO® siRNA Interestingly, the same transfection behavior was observed *in vivo,* using transgenic green mice, with increased particle distribution in bronchial and alveolar regions and at much lower siRNA amounts (EGFP silencing = 68% compared to mismatch group) compared with intranasal administration (N/P = 23). As seen in Figure 2, nanoparticles containing EGFP-specific siRNA, were able to remarkably decrease the fluorescence ratio compared to the mismatch formulation, naked siRNA and the non-treated group. There was no significant difference in the fluorescence ratio between naked siRNA and nanoparticle/siRNA mismatch treated mice [94]. Interestingly, the same siRNA delivery system was proposed as an easy-to-use freeze-dried formulation for potential biomedical applications and longer shelf-life therapeutics. The chitosan-based formulations did not alter their size after lyophilization in the presence of a lyoprotectant (sucrose). Moreover, the induced transfection effect was dependent on the siRNA concentration and on the presence of the lyoprotectant, in EGFP-expressing H1299 cells and proinflammatory cytokine TNF-α expressing RAW murine macrophage cell line. The gene silencing activity of the complexes was retained for up to 2 months when stored at room temperature. Moreover, in contrast to the lyophilized lipid formulation (*Trans*IT-TKO® siRNA), the lyophilized chitosan formulation exhibited higher cell viability [95].

In the context of local therapy, intra-tumoral administration of a chitosan hydrogel loaded with siRNA significantly inhibited tumor growth in melanoma (72%) and breast tumors (92%) in mice, compared to the control [96]. Besides, chitosan/siRNA nanoparticles enhanced docetaxel cytotoxicity and tumor growth inhibitory effect when used as a combination therapy in ovarian cancer cell lines (SKOV3ip1 and HeyA8), where remarkable tumor growth inhibition was observed compared with the control (81.8% reduction in SKOV3ip1, P = 0.017; 84.3% reduction in HeyA8,

$P < 0.05$). These effects were mediated by decreased tumor cell proliferation and angiogenesis, and increased tumor cell apoptosis. Additionally, using chitosan/siRNA systems for dual silencing of Src and Fgr, which are members of the tyrosine kinase gene family over expressed in malignancies, resulted in the greatest reduction in tumor growth *in vivo* (68.8%, $P < 0.05$), compared with silencing of either Src or Fgr alone in the HeyA8 model [97]. Another work elucidated the potential application of chitosan complexes in angiogenesis treatment, using a siRNA targeting vascular endothelial growth factor-A (VEGF-A), which plays a critical role in angiogenesis. *In vitro* transfection of breast cancer cells (MCF-7 and MDA-MB435) revealed the highest (60%) and lowest (29%) gene inhibition, measured in each of the cell lines, respectively [98]. Next, intra-tumoral and intra-peritoneal injections of these complexes were applied to breast tumor-bearing rats. Compared to intraperitoneal injection, the intratumoral led to higher VEGF inhibition which also coincided with higher tumor volume suppression (96%), measured during 36 days. As expected, free shRNA injection resulted in lower tumor suppression [98]. Concerning drug resistance to anti-tumor drugs, chitosan/pshRNA plasmid complexes targeting MDR1 genes were transfected in paclitaxel-resistant ovarian cancer cells (A2780/TS). The complexes exhibited 80-120 nm diameters and could efficiently reverse resistance to paclitaxel in a time-dependent manner, reaching up to 61.3% as evaluated by an MTT assay. This was also consistent with the reduced MDR1 mRNA level of 52.6%, 7 days after transfection [66]. Chitosan/siRNA complexes (114 kDa, DD= 84%) prevented radiation-induced fibrosis by intraperitoneal injection targeting TNF-α in macrophages of mice, without revealing any cytotoxic side effects after long-term administration. Furthermore, TNF-α targeting was selective without a significant influence on tumor growth or radiation-related tumor control probability [99]. In another study, these complexes loaded with FHL2 siRNA (FHL2 belongs to the four-and-a-half-LIM protein (FHL) family which has a role in tumorigenesis) could knock down about 69.6% of FHL2 gene expression in human colorectal cancer Lovo cells, which is very similar to the 68.8% produced when siRNA was transfected with

Lipofectamine®. This specific gene down-regulation resulted in inhibition of cell growth and proliferation [100].

Kong et al. developed a vaccine for the respiratory syncytial virus (RSV), which causes severe bronchiolitis and is deemed a risk factor for asthma. The vaccine is comprised of chitosan (Mw = 110 kDa) complexes containing siRNA targeting RSV-NS1 gene (siNS1), whose protein seemingly antagonizes the host TNF-α. Histological and bronchoalveolar lavage assays showed that the prophylactic use of siNS1 may be an effective method for preventing RSV bronchiolitis, while potentially reducing the later development of asthma associated with severe respiratory infections [101]. On the other hand, chitosan oligomers were administered intranasally to deliver siNS1 in mice. The complexes administered either after or before RSV infection could markedly knockdown the NSI protein, resulting in considerable reduction in virus titers in the lung, as well as in the attenuation of the airway inflammation and reactivity compared to the controls [102].

Several chitosan/siRNA delivery nanosystems were prepared in the presence of the physical cross-linker, TPP, using ionotropic gelation. It is assumed that this agent helps in the reticulation or solidification of the polymeric networks, thus stabilizing the nanoparticulate entity and increasing macromolecule entrapment, as well as ensuring its protection [67]. TPP had been shown to have no effect on transfection efficiency of chitosan-based siRNA nanoparticles as previously reported [27]. On the contrary, *Wang et al.* showed that TPP inclusion, in chitosan/shRNA nanoparticles, resulted in significant EGFP-silencing efficiency (80%) in human embryonic rhabdomyosarcoma cells (RD) compared to chitosan/shRNA complexes (chitosan glutamate, Mw = 460 kDa, DD = 86%), thus being comparable to Lipofectamine® as well [68]. Chitosan nanoparticles could further down regulate TGF-β1 gene, overproduced by RD cells, after subcutaneous injection in nude mice. Notably, modified tumorigenicity resulted in a decrease in tumor volume to 45.5% after 2 weeks of therapy [68].

In support of these findings, in an interesting work, *Katas and Alpar* compared the nanoparticles prepared using two methods of ionic cross-linking, simple complexation and ionic gelation with the physical crosslinker TPP (chitosan glutamate: Mw = 470 and 160 kDa; chitosan hydrochloride: Mw = 270 and 110 kDa; DD = 86%). *In vitro* studies in two types of cell lines (CHO K1 = Chinese Hamster Ovary cells, and HEK 293 = Human Embryonic Kidney 293 cells), revealed that preparation method of siRNA association to the chitosan plays an important role on the silencing effect. In fact, the transfection, carried out in 5% serum, revealed notably better biological activity of siRNA entrapped in chitosan/TPP nanoparticles (82%), compared to either siRNA adsorbed on these nanoparticles (63%) or simply complexed to chitosan (51%) without TPP. In addition to the effect of the preparation method and mixing manner of reagents on the transfection level, the study also highlighted that specific siRNA down regulation was higher after 24 hours than 48 hours. These results could be justified by the higher stability of chitosan nanoparticles, as assessed in 5 and 50 % serum, as well as by the higher protection provided to siRNA, which is prone to degradation when adsorbed on the nanoparticles surface. Surprisingly, chitosan nanoparticles caused transient cell toxicity compared to chitosan complexes, although individual chitosan and TPP solutions exhibited biocompatibility. This is apparently due to the higher chitosan concentration used to transfect the cells in the form of nanoparticles (5.3 μg) compared to that of chitosan complexes (0.53 μg) [67].

Chitosan/TPP nanoparticles were also loaded with siRNA targeting relaxin (RLN), which is a small peptide hormone expressed in several cancers of reproductive and endocrine organs. Increased expression of RLN in prostate cancer correlates with aggressive cancer. RLN behaves as a cell growth factor and increases invasiveness and proliferation of cancer cells *in vitro* and *in vivo.* Intratumoral injection of these nanoparticles, into prostate cancer-bearing mice, resulted in 60% reduction of RXFP1 mRNA at 48 hours post-injection. Excitingly, this treatment effectively suppressed tumor growth and reduced tumor size *in vivo*, being associated with decreased cell

proliferation and increased apoptosis, as well as considerable decrease in metastasis rate in androgen receptor-negative prostate cancer cells (PC3). Furthermore, the suppression of RLN signaling significantly reduced metastasis rates. Global transcriptional profiling of PC3 cells treated with RXFP1-siRNA further revealed genes with markedly altered expression profiles, which have been previously documented to promote tumorigenesis [103]. Ultrafine chitosan/TPP nanoparticles of 20 nm also exhibited excellent transfection efficiency in Neuro2a mouse neuroblastoma cells evidencing feasibility as neurotherapeutics [81]. In another work, chitosan/TPP nanoparticles, loaded with siRNA targeting muscarinic acetylcholine receptor subtypes, were intrathecally injected into rats for 3 consecutive days. Treatment produced a large reduction of the corresponding mRNA levels (50-60%) in the dorsal root ganglion and spinal cord, leading to a marked reduction in the anti-nociceptive effect of muscarine. The encouraging findings attained in this regard paved the road to a new approach of chronic pain treatment in neuronal tissues, using antisense therapy assisted by chitosan nanoparticles [104,105].

5. Nanocarrier-based siRNA delivery using chitosan derivatives

Chitosan poor solubility at physiological pH and its limited buffering capacity are the main incentives that have encouraged research to find alternative derivatives of this polymer. Some of these derivatives display ameliorated solubility within a wide range of pH so that chitosan nanocarriers possess better stability in the physiological environment [16], particularly against nucleases. Furthermore, the use of these derivatives is expected to potentiate *in vivo* transfection kinetics with respect to both efficiency and sustained gene silencing. To this end, structural modifications of chitosan have been explored, such as quaternization of the amine group [27,91] and the introduction of functional groups [82,91] as well as conjugation with other polymers [78] or ligands [78,106]. In this regard, chitosan derivatization has been shown to create safer and more efficient gene vectors [78]. Generally speaking, this strategy has been advantageous not only from

the perspective of drug delivery, but also for broadening the biomedical applications of this polymer [107].

One of the reasons for chemical modification of chitosan lies in the necessity of improving endosomolysis of chitosan/siRNA complexes. Thereby, different strategies have been adopted, such as the use of pH sensitive polyelectrolytes (cationic or anionic) that possess a high proton "sponge effect" [18,108], as well as the inclusion of other compounds possessing an endosomolytic effect [65,90] and the conjugation to some functional groups or polymers [78,91]. For instance, to introduce secondary and tertiary amines into chitosan in order to ameliorate its buffering capacity and solubility at high pH, Mittnacht *et al*. conjugated imidazole to chitosan (Mw = 140 kDa) to deliver siRNA [82]. In addition to cell biocompatibility in a neuronal cell model (PC12 cells), this derivative interestingly manifested homogenous particle distribution in the cell, suggesting efficient endosomal escape. It induced 65-75% degradation of the target mRNA. However, it did not show a better knockdown efficiency than the unmodified chitosan, as previously experienced with pDNA [108]. On the contrary, Ghosn *et al*. used the same vector to deliver siRNA and reported higher gene knockdown compared to native chitosan, which was also equivalent to that of the commercially available siPORT Amines. These outcomes were further corroborated in mice after intranasal administration, where silencing activity reached up to 60% in lung tissue [109].

Trimethylation of chitosan may provide some beneficial characteristics to siRNA delivery, such as increased stability, due to enhanced solubility at physiological pH and an increased positive charge, which assists siRNA cellular uptake while improving the buffering capacity and mucoadhesive properties [110]. Nevertheless, trimethylchitosan (TMC)/EGFP-siRNA complexes, demonstrated low transfectivity in EGFP protein expressing HEK 293 cells at 50 nM of siRNA. This poor transfectability may be accounted for the highly stable complexes in which siRNA disassembly was not facilitated from its carrier. Nevertheless, transfectivity was significantly different from that of native chitosan, which is likely due to the higher positive charge of TMC that

led to enhanced cellular uptake compared to that of chitosan/siRNA complexes [27]. No noteworthy effect for the degree of chitosan quaternization was noticed on the silencing activity, which is consistent with other works [18]. On the other hand, chitosan trimethylation slightly reduced chitosan viability which contradicts other findings in different cell lines [91].

In an effort to promote the endosomolytic activity of TMC, which has a limited buffering capacity, either the membrane-disruptive peptide diINF-7 was added to the formulation or photochemical internalization (PCI) was applied. The latter technique is based on the use of a photosensitizer that locates itself in the endosomes upon incubation with the cells, causing photochemical destabilization of endosomal membranes after illumination, with subsequent release of endocytosed material into the cytosol. The use of both strategies in transfection of luciferase expressing H1299 cells improved the silencing outcome, demonstrating that this chitosan derivative greatly benefited from the enhancement of endosomolysis. This chitosan derivative also showed low cell toxicity, as well as evidenced retained silencing activity in the presence and absence of serum [91]. Besides, the same group suggested a technique for promoting cellular uptake, which does not rely on a surface charge density but rather on the formation of a disulfide bond with mucin glycoproteins on the cell membrane. Thiolation of N,N,N-trimethylated chitosan interestingly demonstrated substantial gene silencing compared to non thiolated counterparts in a dose dependent manner (60-89% vs 40%, respectively), in luciferase expressing H1299 cells (N/P = 8) [90]. Notwithstanding that this finding is in line with other observations [89,111], it contradicts what has been reported that siRNA silencing activity is independent on the siRNA concentration after transfection with chitosan/siRNA complexes using the same cell line (50-200 nM) [65,95]. Likewise, in contrast with non-thiolated formulations, the thiolated counterparts retained their silencing activity even in the presence of hyaluronic acid, illustrating the enhanced stability of these complexes against competitor anionic macromolecules present in the body, certainly, due to thiolation. The enhanced stability and, thus, biological activity after thiolation might be explained

on the basis that the formation of reducible disulfide bonds between thiol groups leads to increased extracellular stability and improved intracellular release properties. On the other hand, the cytotoxicity profile was the same for the thiolated and non-thiolated formulations and more favorable compared to that of the used commercial agent, Lipofectamine®. These findings point out the suitability of thiolated TMC as a gene vector before proceeding to animal experiments [91]. Eudragit® S100, which is a pH sensitive and membrane-destabilizing polyelectrolyte, was also proposed for improving the buffering capacity of TMC. The incorporation of this polyelectrolyte improved transfection efficacy of TMC/siRNA complexes, which could be ascribed to the proton sponge mechanism combined with the endosomal membrane rupture. This may occur as a consequence of complex swelling and/or Eudragit conformational change, mediated by the protonation of its carboxylic group, under the acidic conditions found in the endosome [18].

Notwithstanding the improvement of the endosomolysis attained by the application of the described chitosan derivatives, the endosomolytic property of native chitosan has been verified when the endosomolytic agent, chloroquine, did not affect the silencing level of chitosan/siRNA formulation transfected into EGFP expressing H1299 cells [65]. The obtained gene knockdown was ≈ 78% at 50 nM siRNA in the absence of serum, which was similar to that of thiolated TMC (80%) in the same cell line. The N/P ratio of the complexes and the Mw of chitosan used were quite different in these studies. Despite the difficulty in comparing results, due to differences in the transfection protocols of both studies, this may emphasize the ensodomal escape mechanism endowed by native chitosan, as the chitosan/siRNA complexes achieved good gene silencing in mice after intratracheal administration, as mentioned above [65,91].

Low Mw polyethyleneimine (PEI) (25 kDa) was grafted onto chitosan which was then complexed with shRNA or siRNA resulting in compact and stable complexes that enhanced intracellular uptake in A549 cells. Using a therapeutic shRNA, this chitosan derivative could successfully silence the oncoprotein at both mRNA and protein level, which displayed a better

safety record over the unmodified PEI. This highlights that this conjugation shielded the high positive charge of PEI which is responsible for its known toxicity. Moreover, cell malignancy and metastasis were significantly reduced, as revealed by a soft agar gel assay, and induced apoptosis and reduced cell proliferation were detected [112]. In spite of these outcomes, this system is still lacking cancer cell specificity Thus, to potentiate an *in vivo* application, the same group further conjugated the system with a folate ligand. Most impressively, the folate-chitosan-g-polyethyleneimine vector exhibited better cell viability even at a high concentration, compared to the standard PEI. Moreover, it exhibited enhanced gene transfer efficiency and cancer cell specificity *in vitro*, mediated by folate receptor endocytosis. When investigating the potential of the system *in vivo*, the nebulization of Akt1 shRNA complexes significantly suppressed lung cancer development in urethane-induced lung cancer model mice, suggesting the utility of this carrier for aerosol gene delivery [78].

Another work reported the modification of PEI and glycol-chitosan (GC) with hydrophobic 5β-cholanic acid, afterwards being self-assembled into nanoparticles via strong hydrophobic interactions of 5β-cholanic acid in both polymers [38]. The glycol derivative showed, in previous studies, both a targeting ability and biocompatibility, being used as a carrier for anti-cancer agents [113]. Since siRNA condensation depends on ionic interactions, it was however difficult to condense siRNA with this carrier due to its insufficient charge density, which has been reported to be indispensible for complex formation and stability [16]. Therefore, PEI was employed to confer a strong positive charge, taking into consideration its recognized capability of gene transfection, which is attributed to “proton sponge” effect. The siRNA, complexed with PEI-GC, displayed a 350 nm size and a zeta potential of +23.8 mV, as well as increased biocompatibility, compared to PEI, as assessed in B16F10 tumor cell expressing red fluorescent protein (RFP) (RFP/B16F10). The complexes featured a distinguishable protecting effect to siRNA against RNase degradation at a weight ratio of 5:1 for PEI-GC/siRNA. In these cells, the newly developed complexes showed rapid

time-dependent cellular uptake within 1 hour and a substantial RFP knockdown, estimated as 82% of the cleaved specific mRNA, after 1 day post-transfection and at 200 nM of siRNA. Furthermore, the complexes revealed a high targeting ability to tumor cells in RFP/B16F10-bearing mice, accompanied with significant RFP silencing, following intravenous injection of 50 μg siRNA 3 times every 2 days. In contrast, free siRNA was rapidly excreted from the kidneys within 1 hour post-injection, whereas PEI/siRNA complexes showed non-specific localization by high and rapid accumulation in the spleen and liver, thus being cleared rapidly providing lower knockdown (Figure 3). As expected, these outcomes resulted from the high protection to siRNA in the serum, as well as from the efficient cellular uptake and the endosomal escape provided by the designed carrier [38].

Low molecular weight PEI (25 kDa) was blocked with polyethylene glycol monomethyl ether (mPEG) and, then grafted onto chitosan to synthesize a ternary cationic copolymer for siRNA delivery purpose. Due to both mPEG and chitosan, cell viability of the new vector was ameliorated compared to PEI itself. In comparison to free siRNA, the vector provided protection to the molecule after incubation in 10% serum, particularly at higher N/P ratios. Concerning gene silencing, the transfection of HeLa cells and human Tenon's capsule fibroblasts (HTFs) with 50 nM siRNA resulted in a substantial suppression of mRNA levels of IκB kinase beta (IKKβ) with subsequent inhibition of the nuclear factor kappa B (NF-κB) activation, being associated with the suppression of HTFs proliferation [89]. A subconjunctival injection of this system, at the time of surgery and 7 days post surgery (N/P = 10 and 50 nM), was well tolerated in a monkey model of trabeculectomy. Furthermore, 60 days after surgery, a marked reduction in subconjunctival scar tissue was observed, compared with the eyes of the group treated with phosphate buffer saline. This was also accompanied with a healthy conjunctival epithelium without the acellularity detected in the mitomycin C-treated group (positive control). Due to the ameliorated surgical outcomes, this siRNA complex-mediated blockage of signaling pathway represents a novel approach, which is potentially a more controlled alternative as an anti-scarring agent in glaucoma filtration surgery [111].

Recently, a new multifunctional magnetic nanovector was developed for dual imaging and therapeutic purposes. It is comprised of a superparamagnetic iron oxide nanoparticle core, coated with polyethyleneglycol-grafted chitosan and PEI. siRNA and a tumor targeting peptide, chlorotoxin, were covalently attached to this construct, which constitutes a tool for non-invasive monitorization of siRNA delivery in real-time by means of magnetic resonance imaging. A significant EGFP silencing mediated by the targeted nanoparticles (62%) was observed 48 hours after transfection of EGFP-expressing C6 rat glioma cells, compared to the non-targeted homologues (35%) and other controls. Apparently, PEI inclusion enhanced the endosomolysis and the pegylated chitosan circumvented PEI cytotoxicity. It was then demonstrated that the targeted nanoparticles were internalized through a receptor-mediated mechanism, which can ensure that a greater percentage of cells receive an effective dose of siRNA whilst adsorptive mediated-endocytosis is a non-specific pathway for non-targeted vectors. This clarifies the higher silencing effect of chlorotoxin-targeted nanoparticles [25]. In the context of tracking siRNA mobility during its delivery, the superparamagnetic iron oxide core was also coated with chitosan, conjugated to linoleic acid as a hepatocyte targeting moiety. Interestingly, an *in vivo* study performed in mice using magnetic resonance (MR) imaging, revealed selective accumulation of the nanoparticles in hepatocytes within a few minutes after intravenous injection (Figure 4), these being associated with efficient GFP knockdown and evidencing biocompatibility [106].

Another formulation of self-tracking nanoparticles based on chitosan (200 kDa) was devised. These nanoparticles, encapsulating fluorescent quantum dots (QD), were further conjugated to siRNA targeting human epidermal growth factor receptor 2 (HER2). HER2 antibody and siRNA were conjugated onto the nanoparticles to treat breast cancer (both the delivery and transfection of siRNA can be monitored by the presence of the fluorescent QD in the chitosan nanoparticles). Compared to the non-targeted vehicles, targeted nanoparticles demonstrated considerable gene silencing in both HER2-overexpressing MCF-7 and SKBR3 cells; the effect

being more pronounced in the latter cell line, as these cells express larger numbers of HER2 receptors. This result was also confirmed by the higher cellular uptake, which was receptor-mediated, for the negatively charged and targeted nanoparticles. The study, however, does not tackle the cytotoxicity aspect of this promising delivery system, a relevant detail, as it includes quantum dots [114].

An Arg-Gly-Asp (RGD) peptide was conjugated to chitosan by a thiolation reaction, as another platform for targeted siRNA delivery. The ability of this vector to bind the αvβ3 integrin was examined to ensure its potential targetability. The RGD-chitosan/siRNA complexes significantly increased the selective intratumoral delivery in orthotopic mice models of ovarian cancer. Additionally, targeted silencing of multiple growth-promoting genes (POSTN, FAK, and PLXDC1) along with therapeutic efficacy in SKOV3ip1, HeyA8 and A2780 cell models were verified using this vector. Tumor vascular targeting was further validated *in vivo* by delivering PLXDC1-targeted siRNA into the alphanubeta3 integrin-positive tumor endothelial cells in the A2780 tumor-bearing mice. The developed delivery system enormously inhibited tumor growth compared to the controls [115].

Poly-L-arginine (PLR), a biodegradable cationic polymer, was conjugated with chitosan, which was further pegylated [22]. This approach aims at enhancing, on one hand, the cationic charge of chitosan, thus potentiating its transfectability; and, on the other, improving the cytotoxicity of PLR by conjugation to chitosan. PLR has been described to mediate cellular uptake through its interaction with sulfated proteoglycans and cholesterol [116]. Chitosan complexed to siRNA showed a poor silencing efficiency compared to Lipofectamine^{TM}2000, chitosan-PLR and its pegylated counterpart, although it provided the best cell viability. All complexes composed of chitosan-PLR demonstrated the highest gene silencing either in cells or *in vivo* after intratumoral injection to mice [22]. Considering that stability in serum is a valuable property of delivery systems towards future application *in vivo*, it is important to note that complexes based on the pegylated

chitosan-PLR showed high gene silencing in cells without dependency on serum (N/P = 12), evidencing the best *in vivo* results as well (Figure 5). This higher serum stability could be accounted for by the shielding effect of pegylation, precluding the interaction with serum proteins and RNase digestion.

Another carrier has been reported consisting of chitosan conjugated to thiamine pyrophosphate, which is a water soluble vitamin that plays a role in the cell's energy supply, displaying no toxicity. It assists nanoparticles formation as it reinforces the charge interaction via its negatively charged phosphate group that neutralizes the positive amine of chitosan during complexation, as well as its amine of thiazolium that remains positive even at physiological pH. This property compensates the positive charge loss of chitosan amines at this pH by interacting with siRNA via charged amines of thiazolium and, thus, maintaining the electrostatic interactions that ensure complex stability and, consequently, high gene transfection. This structural modification led to higher EGFP knockdown (70-73%) in EGFP-HepG2 (human hepatocarcinoma) cells compared to the unmodified conventional chitosan lactate (40-50%) at the same mass ratio. This higher transfectability attained at three days post-transfection was comparable with that of the commercial agent, being slightly affected at pH 7.2, which suggests higher stability provided by conjugation with this vitamin. More importantly, this system manifested high cell viability at different Mws of chitosan (20-460 kDa) and chitosan/siRNA weight ratios (0.4-80) [16].

6. Nanocarrier-based siRNA delivery using chitosan in polymeric combinations

Unlike chemical modification of chitosan, researchers have envisaged the design of siRNA vectors through constructing polymeric combinations from chitosan and other polymers, taking advantage of combinatory effects that may substantiate transfectivity. This type of combination is mostly mediated by ionic interactions between chitosan and the other polymer. For instance, siRNA nanoparticles were prepared using chitosan (Mw = 470 kDa, DD = 86%) and polyguluronate (Mw =

6 kDa), which was isolated from alginate (Mw = 200 kDa). The rational for this polymeric combination was to enhance gene transfection through producing lower sized-nanoparticles, as direct complexation between chitosan and alginate often results in large particles owing to the high Mw of both polymers. Interestingly, in this study, all the chitosan-based siRNA nanoparticles featured high cell viability compared to that of Lipofectamine®. In the presence of 20% serum, gene knockdown was greater for complexes containing polygurunate than for those including either alginate or chitosan alone, in HEK293FT and HeLa cells. This greater transfection was comparable to that of Lipofectamine® and poly (L-lysine), which is likely due to the smallest sizes and higher stability of complexes combining chitosan with polygurunate [17].

Chitosan (Mw = 80 kDa, DD = 85%) and poly (γ-glutamic acid) (Mw = 20 kDa) were also combined to deliver siRNA to human fibrosarcoma cells. The rational for this ternary chitosan/ siRNA/poly(γ-glutamic acid) combination was to trigger complex unpackaged as chitosan/siRNA complexes proved to remain stable in the cytosol at pH 7, enabling a later disruption via enzymatic degradation. Relative to the untreated cells, these ternary complexes exhibited considerably higher cellular uptake (96%) than those of chitosan/siRNA (82%); an effect being enhanced with increasing poly(γ-glutamic acid) content in the complexes. This behavior accounts for the significantly higher gene silencing compared to chitosan/siRNA complexes. According to transfection kinetics, the ternary combination expedited siRNA release in the cytosol leading to a faster onset of gene silencing (80% at 48 hours), which was prolonged up to 72 hours. These findings indicate the changes in the mechanism involving transfection after incorporating another polymer into the chitosan/siRNA complex [26].

Another study reported the combination of chitosan-g-PEG with hyaluronic acid via ionic gelation, producing nanoparticles that revealed minimal toxicity and improved stability in 50% serum. Furthermore, they could specifically and efficiently knockdown the Snail transcription

factor, a mediator of tumor invasion, in HEK 293 T cells expressing the fusion EGFP-Snail protein, using two types of siRNA targeted to EGFP and Snail protein [117].

The anionic methacrylic acid copolymer (MAA) (Eudragit® S100) was also employed to prepare an interpolyelectrolyte complex with TMC for siRNA delivery. The presence of MAA did not affect the incorporation of siRNA to the complex, though it led to complex instability owing to charge neutralization. This complex exhibited a larger size, higher cell uptake and mRNA reduction compared to TMC/siRNA complex, measured in murine fibroblasts (L929 cells) 48 hours after transfection (50 nM siRNA). Importantly, it was also concluded that the degree of TMC quaternization did not affect transfection efficiency [18].

7. Nanocarrier-based siRNA delivery using chitosan as an additive material

In order to prevent the loss of the encapsulated material, chitosan has been used as a coating agent or a surface modifier in other delivery nanosystems, benefiting from its high affinity to cell membranes [118]. For instance, poly(lactic-co-glycolic) acid (PLGA) nanospheres, intended for lung siRNA delivery were produced by emulsion solvent diffusion and, thereafter, were coated with chitosan. This modification shifted the nanospheres surface charge to positive values, resulting in higher siRNA loading and increased cellular uptake, mediated by an improved interaction with the negatively charged cell membranes. Notwithstanding that uncoated PLGA nanospheres provide a controlled release [72], chitosan coating further reduced the burst effect from these nanospheres without altering the general siRNA release pattern. This resulted in a sustained release that maintained the silencing activity for up to 5 days in A459 cells. The reduced siRNA release, through chitosan coating, was also confirmed in another work [119]. The higher and more prolonged silencing activity provided by chitosan modification of PLGA nanoparticles could be also accounted for siRNA protection against nucleases [118]. Other authors working with these chitosan-coated PLGA nanoparticles, have found that increasing chitosan concentration led to

increased particle size, siRNA loading and zeta potential, which collectively influenced the silencing activity thereafter. The increment in zeta potential reached a plateau which was attributed to saturated adsorption of chitosan on the nanoparticles' surface. Beyond 0.033% of chitosan coating concentration, stable nanoparticles were formed and protection to siRNA was achieved following incubation with RNase1, which is indicative of effective binding to siRNA. GFP protein knockdown in HEK 293 T cells was demonstrated to be dependent on both siRNA loading and zeta potential, which were also proportionally correlated with chitosan concentration where higher chitosan concentration corresponded to higher positive charge availability to bind siRNA, thus facilitating nanoparticles cellular uptake. These results yielded a silencing effect of 63.3%, in the case of higher siRNA loading, and > 70% for the most positive formulation (+31 mV). In addition, these nanoparticles further demonstrated cell viability [120]. It was also reported the increased PLGA nanoparticles' size with increased Mw of chitosan. The higher Mw of chitosan renders more viscous the organic phase of the process, resulting in the formation of larger emulsion droplets that are more resistant to shear forces. As demonstrated, these particles exhibited biocompatibility in HEK 293 cells regardless of the Mw of chitosan used [121].

siRNA delivery was also studied using core-shell nanoparticles composed of chitosan and poly (isobutylcyanoacrylate) (PIBCA), prepared by radical polymerization, which were designed to target a thyroid cancer oncogene (ret/PTC1). Intratumoral injection of nanoparticles every 2 days in tumor bearing mice resulted in 82% down-regulation for the referred oncogene, significantly inhibiting tumor growth compared to free ret/PTC1 siRNA (Figure 6) [122]. In another work, chitosan-coated poly (isohexylcyano acrylate) (PIHCA) nanoparticles were intravenously injected in breast cancer-bearing mice to deliver the anti-RhoA siRNA, every 3 days at a dose of 150 or 1500 μg/Kg. This treatment inhibited tumor growth by 90% in the group that received the lowest dose and by even more in the highest dose group. Necrotic areas were observed in tumors from animals treated with the highest dose, as a consequence of angiogenesis inhibition. In addition, this

therapy was found to be devoid of toxic effects, as evidenced by similarities between the control and treated animals for the following parameters: body weight gain; biochemical markers of hepatic, renal and pancreatic function; and macroscopic appearance of organs after 30 days of treatment [123]. For the same therapeutic application, other nanoparticles constituted of the monomers, referred to above (PIBCA and PIHCA), used individually, were coated with medium Mw chitosan and administered to mice bearing a papillary thyroid carcinoma. Inhibition of tumor growth by 64% and 59% was observed after intratumoral administration of PIBCA and PIHCA nanoparticles, respectively, at a dose of 1 mg/kg siRNA. Only PIBCA nanoparticles manifested significant tumor growth inhibition after intravenous injection (5 mg/kg siRNA), although they displayed a negative surface charge (-11.8 mV). This effect was attributed to their smaller size (60 nm) and the capacity to escape from macrophage uptake [124].

5. Conclusions and Future Perspectives

From the above mentioned comments, it is clear that chitosan and its derivatives represent excellent biomaterials for the preparation of siRNA nanocarriers. In this respect, special mention should be paid to the structural properties of chitosan, such as molecular weight, degree of deacetylation, salt form, as well as the eventual chemical modifications, that were observed to greatly affect transfectivity of siRNA molecules. Besides, formulation parameters like mass or N/P ratio and pH, have demonstrated to play an important role. All these factors control the physicochemical properties of chitosan nanocarriers in terms of size, surface charge and binding capacity to siRNA molecules, which, in turn, govern transfection kinetics. Nevertheless, there has been no decisive consensus concerning the role of each of these factors on the transfectability of chitosan-based siRNA nanovectors. These discrepancies may arise from differences in the type of cell lines, the preparation methods, as well as the relative abundance of target mRNA and the half-life of its protein, among others. Therefore, it could be inferred that transfection efficiency is the

result of interplay between a number of issues related to the delivery system that should meet the machinery of gene silencing and the characteristics of biological environments. Despite this, binding strength between siRNA and its chitosan-based carrier, which is enormously dependent on mass or N/P ratios, must be investigated in the development of siRNA delivery systems. This binding must ensure efficient balance between the appropriate siRNA protection in biological environments and decoupling in the cytosol, prior to mRNA degradation. However, whilst encouraging results have been achieved in cell culture and animal models, the developed vectors are of limited efficacy *in vivo* and still need to be further adapted to enhance therapeutic output prior to clinical application.

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Figure captions:

Figure 1. Schematic illustration of the structure of chitosan.

Figure 2. Flow cytometric analysis of EGFP knockdown in digested mouse lung after intratracheal administration of aerosolised siRNA formulations in transgenic EGFP mice (n=5, dose; ~0.26 μg in 4 μl on day 1 and 3,tissue harvest day 5). The chitosan/EGFP-specific siRNA nanoparticles (NP/EGFP) showed significant knockdown compared to mismatch nanoparticle controls (NP/Mismatch) and naked siRNA (EGFP) determined by lower EGFP fluorescence ratio, R = (Number of events ×median FL1-H of EGFP-positive population) / (Number of events × median FL1-H of EGFP-negative population). Normalised to non-treated animals set at 100% fluorescence ratio. Error bars: standard error of the mean. P value denotes students t-test ($P<0.05$) analysis of respective groups, Asterix denotes significant between groups (*from [94] with permission*).

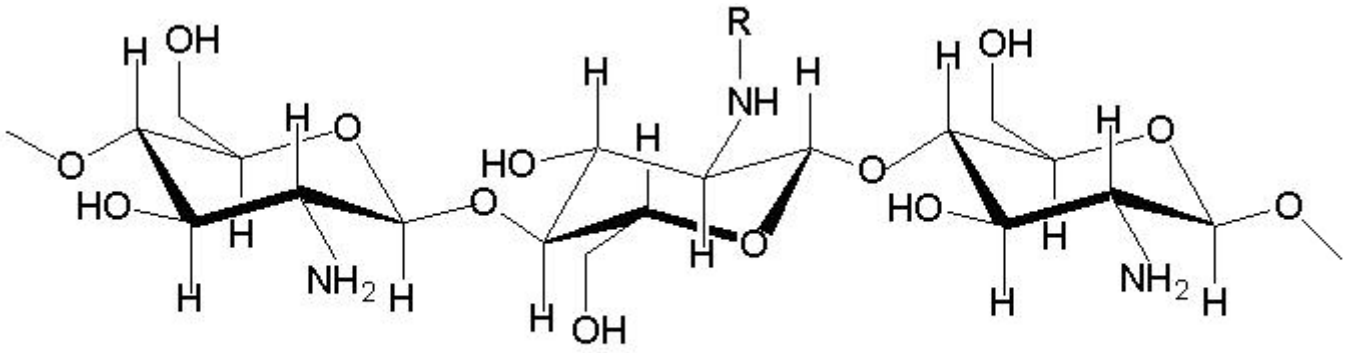
Figure 3. A quantification of *in vivo* targeting characteristics of Cy5.5-siRNA and Cy5.5-siRNA–GC–PEI NPs was recorded as total photons per centimeter squared per steradian (p/s/cm^2/sr) per milligram of each organ at all time points (n=3 mice per group). All data represent mean ± s.e. (*adapted from [38] with permission*).

Figure 4. Nuclear images (A) of mice at 30 min and 1 h after injection of ^{99m}Tc-labeled SCLNs. MR images (B) of the middle part of the mouse liver before and after injection of SCLNs (*from [106] with permission*).

Figure 5. Quantitative expression of RFP in siRNAs-treated mice tumors relative to untreated tumors after 3 days of intratumoral administration of: naked siRNA, chitosan-PLR (CS–PLR) and pegylated chitosan-PLR (PEG–CS–PLR) complexes. siRNAs (siRFP and siGL2) were administered to the right flank of mice bearing B16F10-RFP tumors whereas tumors in the left flank were untreated as a control (n = 4, *: significantly different from the group treated with siGL2 delivered by the same carrier; $P < 0.05$) (*adapted from [21] with permission*).

Figure 6. Antitumor effect of the ret/PTC1 small interfering RNA (siRNA) in the RP1 graft. Injections were performed on days 0, 2, 4, 7, 10 as indicated by the arrows. siRNA were dissolved in 0.9% NaCl. The following preparations were intratumorally injected: free siRNA #1 (siRNA #1), free control siRNA (Ctrl siRNA), unloaded nanoparticles (free NP), siRNA #1 associated with nanoparticles (siRNA #1/NP) and control siRNA associated with nanoparticles (Ctrl siRNA/NP). $P < 0.05$ versus 0.9% NaCl-treated mice) (adapted *from [122] with permission*).

Figure 1



R = H or $COCH_3$

Figure 2

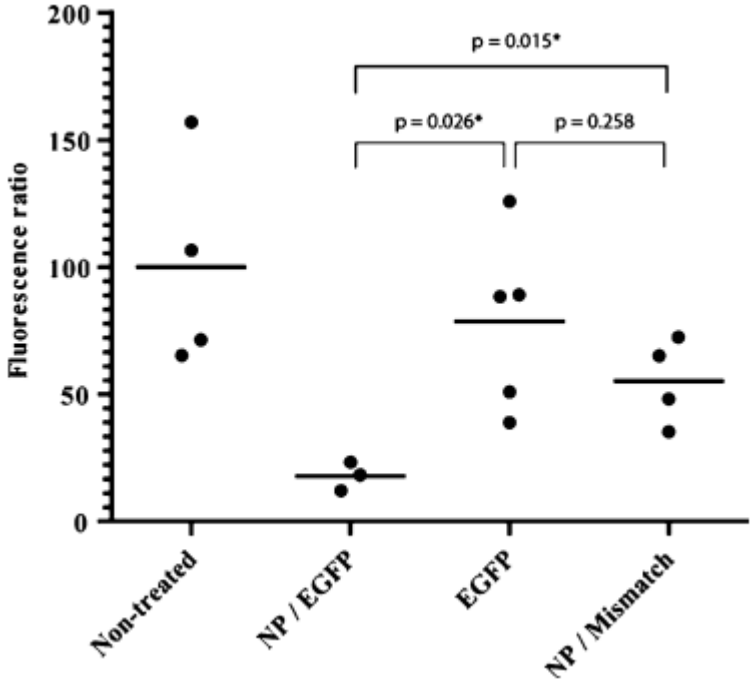

200
150
100
50
0
Fluorescence ratio
p = 0.015*
p = 0.026*
p = 0.258
Non-treated
NP / EGFP
EGFP
NP / Mismatch

Figure 3

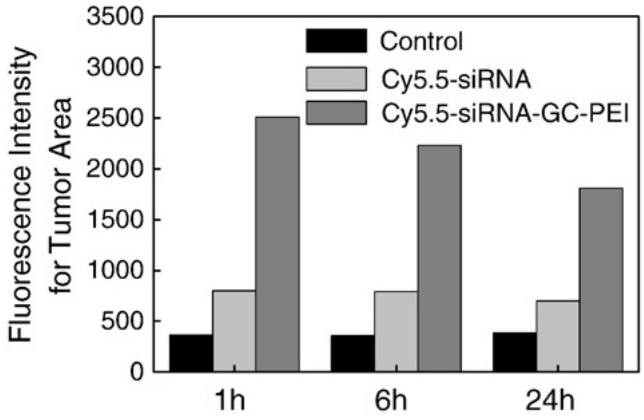

Fluorescence Intensity for Tumor Area
3500
3000
2500
2000
1500
1000
500
0
Control
Cy5.5-siRNA
Cy5.5-siRNA-GC-PEI
1h
6h
24h

Figure 4

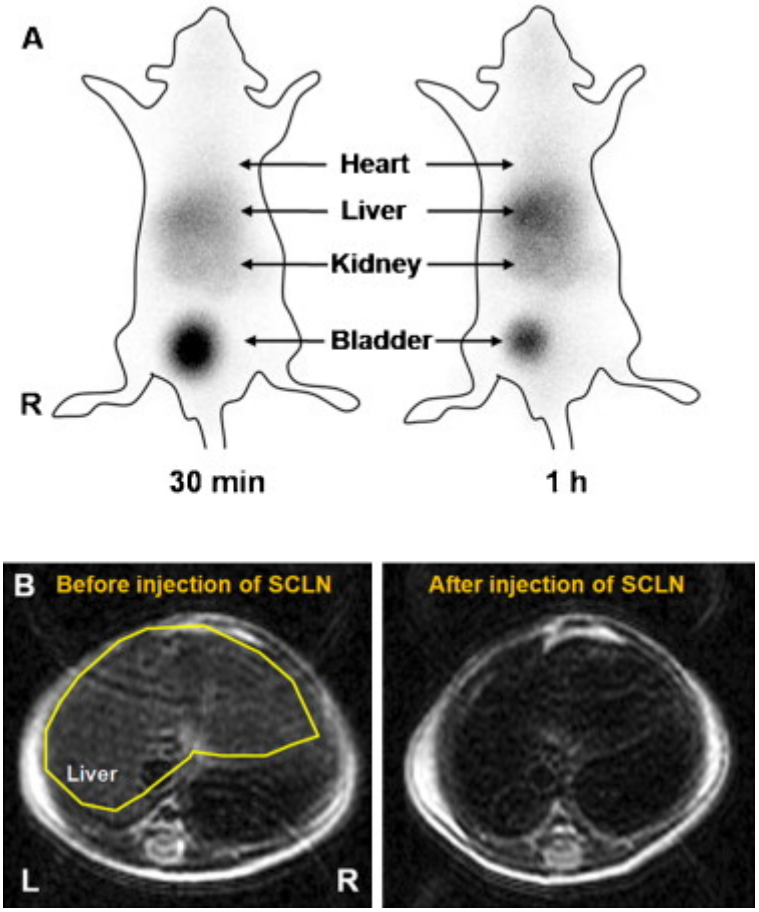

A
Heart
Liver
Kidney
Bladder
R
30 min
1 h
B
Before injection of SCLN
After injection of SCLN
Liver
L
R

Figure 5

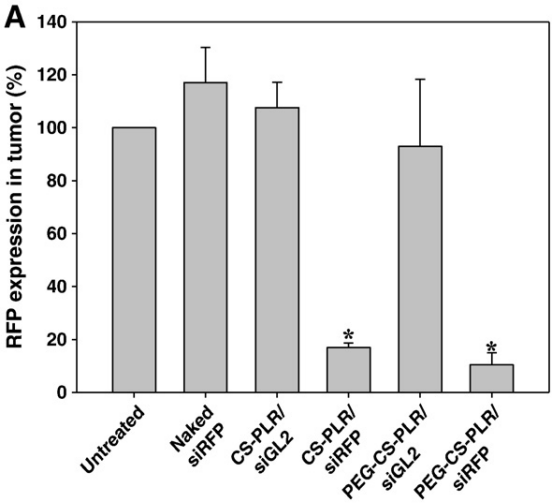
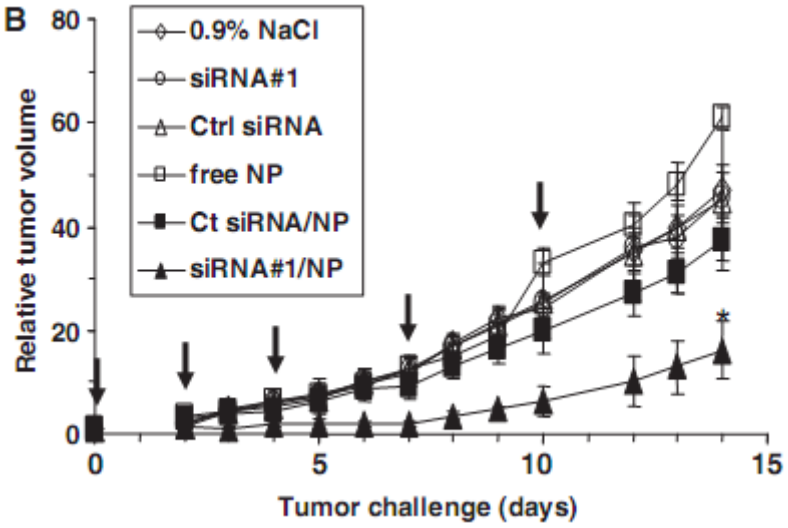

A
RFP expression in tumor (%)
140
120
100
80
60
40
20
0
*
*
Untreated
Naked siRFP
CS-PLR/ siGL2
CS-PLR/ siRFP
PEG-CS-PLR/ siGL2
PEG-CS-PLR/ siRFP

Figure 6


B
80
60
40
20
0
Relative tumor volume
0.9% NaCl
siRNA#1
Ctrl siRNA
free NP
Ct siRNA/NP
siRNA#1/NP
*
0
5
10
15
Tumor challenge (days)