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Review

Nanocarrier mediated delivery of siRNA/miRNA in combination with chemotherapeutic agents for cancer therapy: Current progress and advances

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ABSTRACT

Chemotherapeutic agents have certain limitations when it comes to treating cancer, the most important being severe side effects along with multidrug resistance developed against them. Tumor cells exhibit drug resistance due to activation of various cellular level processes viz. activation of drug efflux pumps, anti-apoptotic defense mechanisms, etc. Currently, RNA interference (RNAi) based therapeutic approaches are under vibrant scrutinization to seek cancer cure. Especially small interfering RNA (siRNA) and micro RNA (miRNA), are able to knock down the carcinogenic genes by targeting the mRNA expression, which underlies the uniqueness of this therapeutic approach. Recent research focus in the regime of cancer therapy involves the engagement of targeted delivery of siRNA/miRNA in combinations with other therapeutic agents (such as gene, DNA or chemotherapeutic drug) for targeting permeability glycoprotein (P-gp), multidrug resistant protein 1 (MRP-1), B-cell lymphoma (BCL-2) and other targets that are mainly responsible for resistance in cancer therapy. RNAi-chemotherapeutic drug combinations have also been found to be effective against different molecular targets as well and can increase the sensitization of cancer cells to therapy several folds. However, due to stability issues associated with siRNA/miRNA suitable protective carrier is needed and nanotechnology based approaches have been widely explored to overcome these drawbacks. Furthermore, it has been univocally advocated that the co-delivery of siRNA/miRNA with other chemodrugs significantly enhances their capability to overcome cancer resistance compared to naked counterparts. The objective of this article is to review recent nanocarrier based approaches adopted for the delivery of siRNA/miRNA combinations with other anticancer agents (siRNA/miRNA/pDNA/chemodrugs) to treat cancer.

Contents

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1. Introduction

Cancer is a leading cause of death and according to World Health Organization accounted for almost 8.2 million deaths worldwide in 2012 [1]. Lung, breast, prostate, pancreatic, stomach, liver, and colon cancer are leading causes of cancer deaths around the world. Of all the cancer related deaths, lung cancer is the leading cause worldwide, accounting for around 1.59 million deaths in 2012 followed by liver (745,000), stomach (723,000), breast (521,000) [2]. The current therapies for cancer treatment include chemotherapy, radiotherapy and surgery. Chemotherapy continues to play an important role in treatment of cancer, despite several advances in the field of surgery and radiotherapy [3].

Chemotherapy involves the use of chemotherapeutic drugs to inhibit or control the growth of cancer cells [4,5]. The cytotoxic agents however pose many limitations that may result in reduced effectiveness of the chemotherapeutic agents [6–8]. The non-selective nature of most of the therapeutic agents results in significant damage to the normal cells. These agents also lack specific distribution in the body resulting in insufficient penetration into the tumors causing toxicity to normal healthy tissues and further limiting the dose and or frequency of dosing [9,10]. Another important limitation associated with chemotherapeutic drugs is the emergence of multidrug resistance (MDR) and is mainly the result of two mechanisms viz. the drug efflux pumps on the cell membrane and augmented anti-apoptotic mechanisms [11–13]. The development of MDR in cancer cells due to increased efflux pumps leads to a decreased intracellular concentration of drug ultimately resulting in the failure of chemotherapy [9,14,15]. On the other hand, the anti-apoptotic mechanism developed by cancer cells enables them to survive against the cytotoxic effect of chemotherapeutic agents [16,17]. The one dimensional action mechanism of single drug therapy often leads to the activation of alternate pathways resulting in development of chemo resistance and tumor relapse [18,19].

Combination therapy has been recommended for the treatment of cancer due to its primary advantage of increased efficacy due to additive or synergistic anticancer activity [20,21]. It is possible to achieve the synergistic effect with the use of appropriate combination of chemotherapeutic agents which improves the therapeutic outcome and patient compliance due to reduced dose and decreases development of cancer drug resistance [18,22,23]. RNAi mediated by siRNA and miRNA has emerged as one of the most promising strategy for anticancer therapy. Nucleic acid based bioactive such as siRNA that can potentially down regulate the gene expression has shown huge promise under *in vitro, in vivo* and clinical trials for the treatment of cancer [24]. The potential advantage of siRNA strategy includes target specificity and ability to inhibit the expression of a mutant carcinogenic protein without affecting the wild type [25,26]. MiRNA is another potentially vital group of nucleic acid based agents that has enormous potential to be developed as an anticancer therapeutics [27–29]. MiRNAs have been shown to play a very important role in various cellular processes such as apoptosis, development and differentiation. MiRNAs also have been shown to be mis-expressed in cancers and exert their effect as oncogenes or tumor suppressors [30].

The objective of this article is to review various nanoformulation approaches that have been adopted to deliver widely studied siRNA and recent miRNA based combinations with chemotherapeutic drug for cancer therapy. It is anticipated that this article will give an update to formulation scientists about the progress done towards development of siRNA/miRNA based combinations.

2. RNA interference (RNAi)

RNAi is a natural mechanism occurring in most eukaryotic cells in which the double stranded ribonucleic acids (dsRNAs) undertake the function of regulating gene expression [31]. It is a specific regulatory mechanism, which helps in regulating various biological pathways and protecting the body against various pathogens [32,33]. RNAi represents a novel way to treat diseases, which would not have been possible with the conventional medicines [34]. The RNAi based medicine involves delivery of double stranded siRNA or miRNA to the diseased cells [31]. The RNAi sequences can be easily designed to target the specific genes. One of the important use RNAi based medicine is to target some of the proteins which are involved in certain diseases and cannot be targeted using conventional molecules, due to the lack of enzymatic function or inaccessibility. Such non-druggable targets have been easily targeted using siRNA/miRNA [31]. The two main types of RNAis, siRNA and miRNA have been described in brief in the following sections.

2.1. Small interfering RNA

SiRNAs are chemically synthesized duplex which are 19–23 nucleotide (nt) long having 2-nt-3′ overhang, comparable to that of endogenous miRNAs. This allows them to be easily recognized by the enzyme DICER and undergo further processing. The duplex siRNAs are then unwound by helicase activity of Argonaute. One of the two strands, a guide strand is retained within the complex RNA inducing silencing complex (RISC) while the other passenger strand undergoes degradation by exonucleases. The RISC-siRNA complex then leads to degradation of mRNA. The detailed mechanism of siRNA interference is explained in Fig. 1 [31].

2.2. Micro RNA

MiRNA are 20–24 nucleotide long, double stranded, endogenous RNA molecules which also plays important role in regulating gene expression [35,36]. MiRNA are involved in mediating the post-transcriptional

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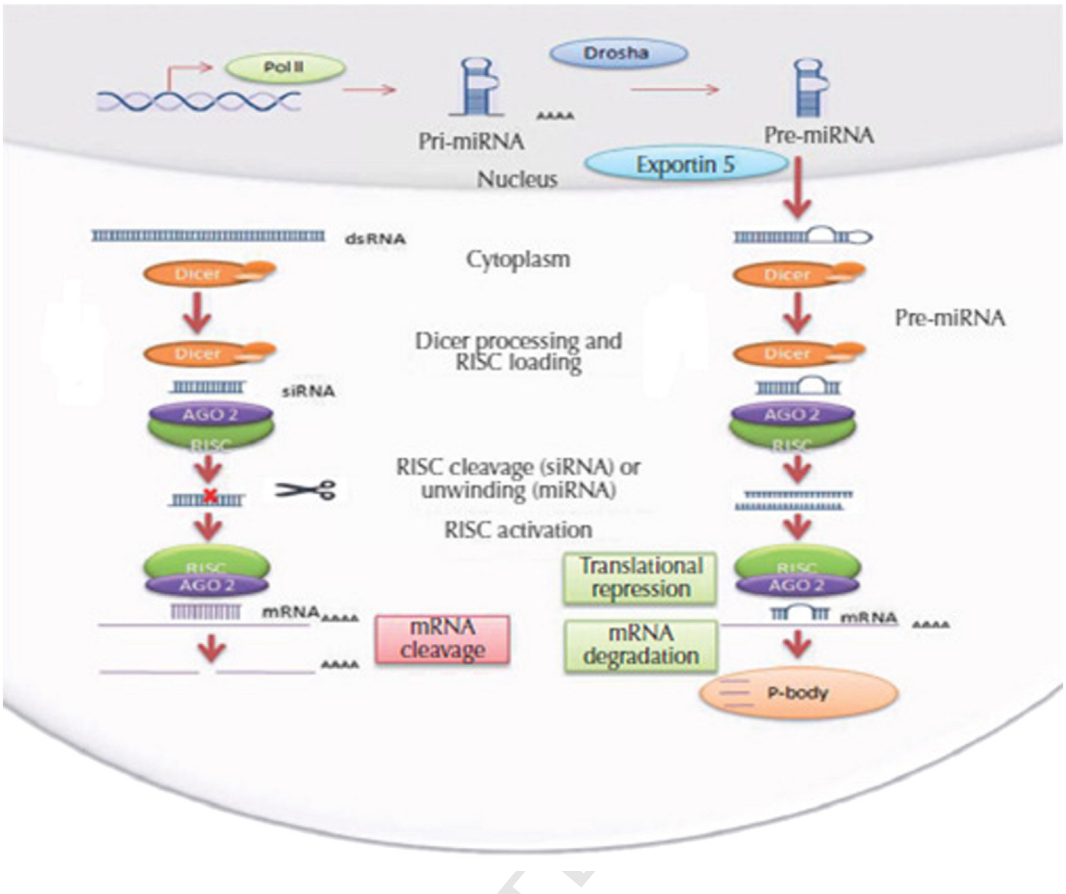

Fig. 1. RNA interference mechanism: siRNA: The siRNA pathway begins with cleavage of dsRNA by enzyme DICER resulting in siRNA in the cytoplasm of cell [34,49]. The siRNA then binds to Argonaute (AGO2) protein and RNA inducing silencing complex (RISC) [37]. One strand of the siRNA duplex (the passenger strand) is removed by AGO2 resulting in RISC containing guide strand [50]. The activated RISC-siRNA binds to the complementary sequences on the mRNA and results in its cleavage and degradation [51]. Biogenesis of miRNA: The RNA polymerase II or III is responsible for the production of primary-miRNAs (pri-miRNA) [36,52]. In the nucleus, the resulting pri-miRNAs are cleaved by the microprocessor complex Drosha [53]. The pre-miRNA is transported to the cytoplasm by Exportin 5 (XPO5) and the loop structure is removed by the Dicer complex (Dicer–TAR binding protein) resulting in miRNA or miRNA duplexes [54,55]. One strand of the duplex is incorporated into AGO2 and RISC which targets mRNA and results in its degradation [56]. (Adapted with permission from [57]).

silencing of genes [37]. miRNA is capable of controlling the expression of more than one mRNA, a distinguishing feature from siRNA [38]. The biogenesis of miRNA begins with transcription by RNA polymerase II or III producing primary miRNA (pri-miRNA) in the nucleus, which is further processed by Drosha and the DiGeorge critical region 8 (DGCR8) to yield a long nucleotide. It is transported to the cytoplasm where it is processed further and similar to siRNA, forms an active complex with RISC. This complex then binds to the mRNA leading to its degradation. Fig. 1 illustrates the detailed biogenesis pathway of miRNA.

SiRNA/miRNA induces the gene specific cleavage through its complementary pairing with mRNA and resulting in degradation of mRNA. SiRNA/miRNA has the ability to knock down genes and overcome the cellular pathways and help treat diseases caused by aberrant gene expression [39,40]. Results have been promising with the use of siRNA to knock down the genes related to MDR mechanisms and improve the sensitivity of resistant cancer cells to chemotherapeutic agents [9,41]. Hence, the sensitivity of cancer cell to chemotherapeutic agents can be enhanced using combination therapy with siRNA which will help to prevent the development of chemo resistance [42,43]. Simultaneously inhibiting multiple targets using siRNAs of different nature and origin is also an effective approach to treat cancer [43]. On the other hand it has been found that miRNAs also play a very crucial role in tumorigenesis and drug resistance [44]. A single miRNA has the potential to bind to thousands of mRNA and can either act as a tumor suppressor genes when down-regulateded or as an oncogene (oncomirs) when up-regulated [45]. MiRNA have also been shown to be implicated in cancer stem cells (CSCs) and epithelial–mesenchymal transition (EMT), which are critically associated with cancer metastasis and drug resistance [46].

The pathogenesis of tumor is heterogeneous and progression occurs due to the defects in various signaling pathways associated with tumor tissues. The tumor cell signaling pathways primarily involves interaction of growth factors with receptors e.g. human growth factor receptor, insulin-like growth factor receptor, etc., and thereby resulting in downward cascade of signaling [47]. In certain cancer such as non-small cell lung cancer (NSCLC), activation of oncogenes and growth factor signaling plays a very decisive role and using different therapeutic siRNAs to target molecular targets involved in tumor development can significantly reduce the tumor growth [48]. Angiogenesis is also an important process in progression and growth of tumor tissue. Based on specific pathways involved in the cancer progression, the rationale selection of siRNA or miRNA in combination with chemodrug will provide effective treatment options. The siRNA and miRNA have similar properties such as negative charge, instability in serum and cytosol as delivery target site. The therapeutic concentration of miRNA or siRNA in tumor tissue is required to elicit the anticancer effect and hence, the optimization of nanoparticles in term of size, charge, release, stability, pharmacokinetic and pharmacodynamics properties needs to be performed [48]. Considering some of the above mentioned factors and other such factors discussed later in the article, an appropriate nanoparticle system can be selected to deliver the agents.

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3. Problems with *in vivo* delivery of siRNA and miRNA

3.1. Biological instability

The short lived nature of siRNA and miRNA's gene silencing effects along with their poor stability in biological systems is one of the major obstacles towards their successful application as therapeutic agents [58,59]. The siRNA/miRNA are rapidly degraded by endo- and exonucleases and quickly eliminated by kidney filtration due to their low molecular mass (~13 kDa) [60,61].

Various strategies such as chemical modifications of the backbone, glycation, nucleic acid locking, etc., have been investigated to improve their stability under biosystems [59,60]. However, aforementioned motifs of attaining biological stability have its own allied limitations [62,63], and hence successful use of siRNA/miRNA in cancer therapy demands alternative approaches that can protect them from adverse environment while retaining their bioactivity without concomitant activation of immune system.

3.2. Stimulation of innate immune system

Long dsRNA has the ability to trigger sequence specific innate immune system that primarily involves the activation of interferon (IFN) system [64,65]. DsRNA was found to induce IFN responses by binding to dsRNA activated protein kinase (PKR), 2′,5′-oligoadenylate synthetase- RNase L system retinoic acid-inducible gene I (RIG-I) or several Toll-like receptors (TLRs); which are mostly aimed at combating viral pathogens [66,67]. These outcomes direct the need to explore a delivery system that can protect the exposure of such codes and prevent initiation of immuno responsive elements within the body (i.e. to avoid 'off-target effect'). At the same time, it must be noted that such delivery system must be capable to concomitantly deliver these bioactive at desired site of action.

3.3. Off-target effects

Although originally thought to be highly specific, but similar to miRNA, siRNA also has the ability to regulate large number of transcripts [68,69]. The off targets effects are generally prominent when there is a match between the seed region of siRNAs (positions 2–7) and sequences in the 3′ UTR of the off-target gene. There are several reported modifications of siRNA that have shown to eliminate off-target effects such as phosphorothioate or boranophosphate introduction, modification of the 2′- position, etc. Thus, in order to minimize the off-target effects of siRNA several factors such as dose, backbone design and structural modification must be taken into consideration [70].

4. Rationale behind adoption of RNAi based drug combination therapies

Combination therapy with siRNA or miRNA significantly enhances the sensitivity of chemotherapeutic drugs by sensitizing the genes involved in developing the chemotherapeutic resistance [71]. Before going into further details of strategies dealing with the delivery of RNAi based chemo-combination, it is imperative to understand the key mechanisms by which cancer cell attains chemoresistance. There are two key mechanisms viz. efflux pump and non-efflux pump by which the tumor cells are more likely to develop chemo/drug resistance. Following section briefly discusses these two mechanisms.

4.1. Emergence of cancer drug resistance: Mechanistic outlook

4.1.1. Membrane transporters or efflux pump alterations

Efflux pump alternation is the expression of an energy-dependent drug efflux pump, known alternatively as P-gp or the multidrug transporter (Fig. 2) (14, 15). MDR-1 gene is primarily responsible for activating the efflux pump. Other related genes such as MDR-1a and MDR1b are also involved in similar activation process. P-gp efflux pumps are one of the first members of adenosine triphosphate (ATP)-dependent transporters family known as the ATP-binding cassette (ABC). The P-gp efflux pumps are usually present on the cell membrane and/or the nuclear membrane and possess the capability to bind either to positive or neutrally charged molecules. It may be noted that majority of chemotherapeutic drugs are either neutral or positively charged under extra- or intra-cellular pH, and thus acts as a substrate for P-gp pumps. Hence, after encountering P-gp pump, chemotherapeutic drugs can be pumped out of the cell leading to a decreased effective concentration inside the cellular compartment [9,72]. This mechanism can be thus stated as self-defense machinery, mainly exhibited by the cancer cells to protect them against the cytotoxic action of chemotherapeutic drugs. In addition to this mechanism, cancer cells also activate antiapoptic pathways as a protective mechanism.

4.1.2. Activation of anti-apoptotic pathways: A key cancer resistance conduit

Apoptosis is most common type of programmed cell death, which is also very vital for embryogenesis; tissue homeostasis and defense against pathogens [73,74]. The activation of anti-apoptotic pathways is yet another key defense mechanism that rescues cells from cell death. A series of cascade signals activate apoptosis involving several proteins. B-cell lymphoma-2 (BCL-2) is among the first apoptotic regulator to be identified. Bcl-2 protein is encoded by the gene BCL-2 and it belongs to Bcl-2 family, which has a major role in preventing apoptosis in healthy cells by promoting cell survival rather than by driving cell proliferation and it is correlated with cancer cell survival and resistance (Fig. 2). Myeloid cell leukemia-1 (Mcl-1), a protein encoded by the gene MCL-1, is another member of the class of BCL-2 that has been identified as an inhibitor of apoptosis and inducer of drug resistance by BCL-2 family [9,75]. This article is mainly focused on the siRNA and miRNA based delivery systems in the treatment of cancers. The drug resistance mechanism is explained in detail elsewhere [72,76].

4.1.3. Strategies to overcome cancer resistance using RNAi based chemotherapeutic drug combinations

There are several strategies employed recently to overcome both in efflux and non-efflux pump related MDR in the developed by cancer cells [77,78]. Sensitization strategies using siRNA to knock down the primary efflux pump receptors genes, encoding for proteins such as P-gp, MRP have shown huge promise. Meng et al. synthesized silica nanoparticles containing combination of siRNA against P-gp pump and doxorubicin (DOX) to sensitize the DOX resistant KB-V1 cervical cancer cells. Investigators studied the down regulation of the genes associated with the activation of P-gp pump using siRNA. This strategy navigated the cancer cells from resistant stage to sensitized stage and the delivery of higher intracellular concentration of DOX resulted in increased anticancer activity [79].

Several sensitization strategies have been employed to overcome non-efflux pump related MDR [80]. Strategies include inhibition of cell survival pathways, altering transcription factors and silencing anti-apoptotic factors using siRNA [9]. Cationic micelles have been used to deliver siRNA targeting BCL-2 and docetaxel (DTX) *in vivo* to investigate the synergistic tumor suppression effect against breast cancer [81]. Trilysinoyloleylamide based liposomes have also been used to deliver anticancer drug suberoylanilidehydroxamic acid and siRNA targeting gene encoding for Mcl-1 protein involved in anti-apoptotic defense mechanisms against human epithelial cancer [82]. Other such promising approaches using siRNA in combination with chemotherapeutic agent to overcome both efflux and non-efflux pump related genes for effective treatment of cancer have been reviewed in detail in later sections.

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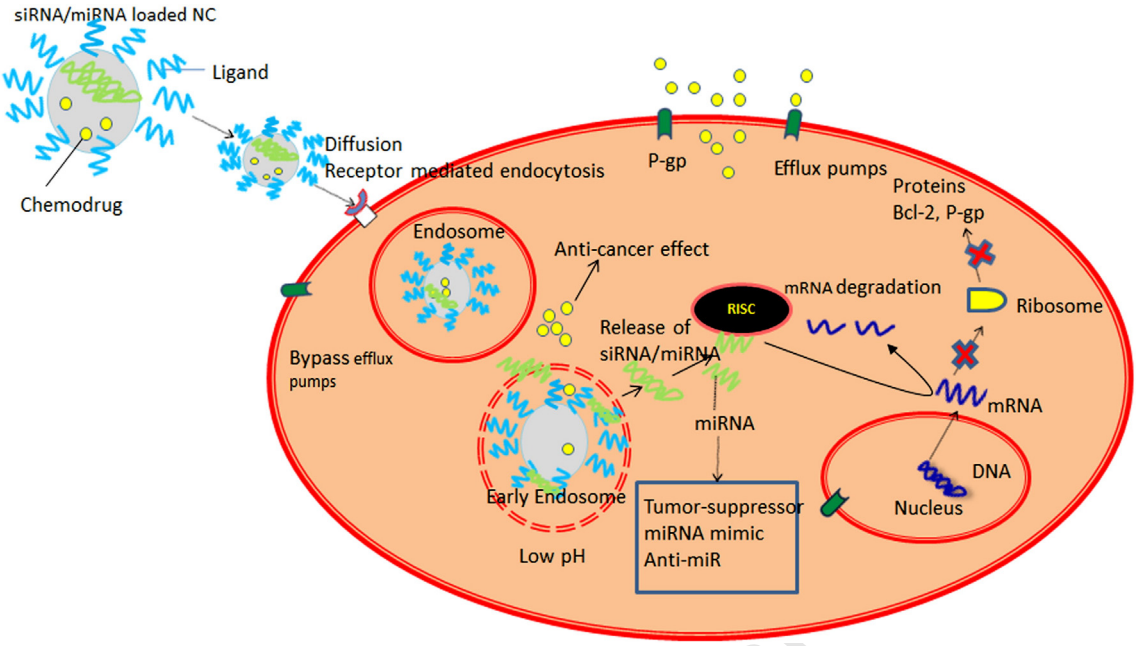

Fig. 2. Mechanism of sensitization of resistant cancer cells by co-delivering siRNA and a chemotherapeutic agent. Therapeutic agents encapsulated in nanoparticles evade the efflux pump via endosomal internalization. Once in the endosome, the specifically designed nanoparticles release siRNA/miRNA and drug in the cytosol resulting in the cytotoxic effect.

4.2. Tumor angiogenesis: Rationale for using RNAi based combination

Experimental evidence suggests that tumor growth and metastasis is also dependent on the angiogenesis, a process of formation of new blood vessels [83,84]. The tumor after attaining a very small size further develops new blood capillary networks to facilitate further tumor growth [85]. Specific macrophages and certain angiogenic molecules are involved in formation of new blood vessels [86,87]. The switch to angiogenic activity generally involves two stages—the prevascular and the vascular phase [88,89]. There is a limited tumor growth in prevascular phase, which may persist for several years, while the vascular phase is usually associated with the rapid tumor growth with a high risk of metastases [90,91].

In the event of tumor progression and metastasis, vascular endothelial growth factor (VEGF) is yet another potent pro-angiogenic factor. The inhibition of the activity of VEGF leads to the suppression of various factors that cause tumorigenesis viz, proliferation of endothelial cells, angiogenesis and tumor growth. Recently, various chemotherapeutic agents along with siRNA targeting VEGF gene have been explored with high positive effects [48,92,93].

It is evident that the siRNA/miRNA are potential tool in a researcher's armory for the treatment of cancer. However, the delivery of siRNA/miRNA is still challenging and research efforts have been ongoing to improve the delivery to tumor tissues. In this meadow, nanotechnology based strategies represents promising mode to deliver siRNA/miRNA in combination with chemotherapeutic drug to attain additive or synergistic effect. Following section presents various nanotechnology based approaches employed to deliver siRNA/miRNA in combination with chemotherapeutic drug in the treatment of cancer.

5. Nanotechnology based approaches to deliver RNAi based combinations

Nanotechnology is a multidisciplinary field covering various areas from biology, engineering, chemistry and physics [94,95]. Nanotechnology based therapeutics typically includes nanosized particles composed of different entities such as lipids, polymers, inorganic materials, etc. [96,97]. The term nano assembly is usually given to architect the range in their diameter in the size range of 10 to 200 nm [98]. The enhanced permeability and retention (EPR) effect is a property of tumor tissue which allows nanoscale molecules or particles to accumulate in the tumor tissue compared to normal tissues. Typically for the successful employment of the prolonged circulatory lifetime and enhanced permeation and retention (EPR) effect, nanoparticles of 20–100 nm are recommended [99,100]. However, nanoparticles of <20 nm undergo clearance via hepatic and renal routes of elimination. The tumor vasculature has a pore cutoff size between 380 and 780 nm [101]. Surface charge is also an important factor which determines the stability and biodistribution of the nanoparticles inside the body [102]. For example, it has been reported that cationic and anionic liposomes activate the complement system through different pathways compared to the neutral charged liposomes [103]. Recently, Xiao et al. have reported that a slight negatively charged nanoparticles (around −8.5 mV) helped in reducing the liver uptake, prevent aggregation in the blood and deliver anti-cancer drugs more efficiently to the tumor cells compared to the positive and negative counterparts [102]. The variable results might be due to the inconsistent particle sizes, different types of nanoparticles and the varying nature of the surface charges. These studies suggest that the nanoparticle surface property needs to be optimized for the surface charge to achieve an enhanced intratumoral delivery.

Reticuloendothelial system (RES) including liver, spleen and other parts are responsible for clearing the nanoparticles from the system [104]. Apart from the criteria of having particle size approximately 100 nm and optimized surface charge, another important property the nanoparticle should possess is the hydrophilic surface which reduces the clearing from RES system [105]. The attachment of polyethylene glycol (PEG) on the surface of nanoparticles helps significantly in reducing the RES uptake and increases the circulation lifetime of the nanoparticles compared to the uncoated nanoparticles . The aggregation of nanoparticles also reduces significantly as PEGylation helps avoiding the interaction with serum and tissue proteins [106].

The potential advantages of nanotherapeutic strategy includes : (a) higher delivery of loaded therapeutic agents, (b) can be delivered through various routes of administrations including oral and inhalation, and (c) can be used to deliver both hydrophilic and hydrophobic therapeutic moieties. The intravascular deliverable nano-vectors represent the major class of nanotechnology based systems used to deliver therapeutic agents for cancer therapy. Various carriers such as liposomes

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[107], polymers poly (D,L-lactide-co-glycolide) (PLGA) [108,109], poly lactic acid (PLA) [110,111], poly capro lactone (PCL) [112–114]), dendrimers [115,116], and silica [117–119] have been used to deliver the siRNA based combinations to treat cancer. The miRNA based combination therapies are in its early stage of development. Various carriers such as cationic lipoplexes [120], polyethylenimine (PEI) bound to iron oxide magnetic nanoparticles (MNP) [121], PLGA [122] have been used to deliver miRNA for cancer therapy. The following section of article systematically reviews the work done in the field of nanocarrier based approaches for the delivery of RNAi based combinations.

5.1. Inorganic nanoparticles based siRNA combinations

Inorganic nanoparticles represent an efficient alternative due to the lower toxicity [123] and also can be modeled to possess the controlled release properties [124]. In perspective of drug delivery, bioactives can be incorporated inside inorganic nanoparticulate systems without any chemical modifications of bioactives [125]. The inorganic nanoparticles that have been used for delivery of siRNA/DNA comprise of silica, calcium, gold, magnesium, strontium, quantum dots, etc. [126]. Inorganic nanoparticles possess several versatile properties suitable for the cellular delivery including biocompatibility, controlled release of therapeutics agents, and capability of targeted drug delivery. The inorganic nanoparticles can be used for various routes of administration including nasal, parenteral, intra-ocular, etc. The inorganic nanoparticles possess ability to accumulate in cells without being recognized by P-gp, one of the main mediators of MDR, resulting in the increased intracellular concentration of drugs [127]. The various siRNA and chemotherapeutic agent combinations delivered using inorganic nanoparticles are discussed below.

One such inorganic material mesoporous silica based nanoparticles (MSNs) has been widely investigated as carriers for the targeted drug delivery system [128,129] (Table 1). Apart from being chemically stable, it is safe, biocompatible and biodegradable [130,131]. MSNs possess several advantages over other inorganic carriers such as having large pore volumes to encapsulate higher amounts of drugs along with the property of improved stability associated with their inorganic oxide framework [132]. It has also been observed that MSNs can easily escape the endolysosomal compartment and release the content in the cytoplasm [133,134]. Thus, MSNs are capable of releasing the content into the cytoplasm along with serving as delivery vehicles.

Taratula et al. have developed a lung tumor targeted drug delivery system (DDS) based on MSN [135]. The MSN carrier was used to co-deliver anticancer drugs [DOX or cisplatin (CIS)], suppressor of pump resistance (siRNA targeting MRP-1 mRNA), and suppressor of non-pump cellular resistance (siRNA targeting BCL2 mRNA) using tumor targeting moiety luteinizing hormone releasing hormone (LHRH) peptide. The fluorescence microscopy and RT-PCR studies revealed efficient intracellular delivery of DOX and successful release of siRNA in cytoplasm. The half maximal inhibitory concentration (IC_{50}) dose of MSN based DDS carrying DOX and CIS (IC_{50} = 1.5 μg/ml) was five times higher compared to LHRH targeted MSN-drug complexes carrying both BCL2 and MRP1 siRNA (IC_{50} = 0.3 μg/ml). The inhalation delivery of LHRH targeted MSN-drug complexes carrying both BCL2 and MRP1 siRNA (LHRH-PEG-siRNA-DOX-MSN) showed that 73.6% of MSN was retained in lung compared to 5% when intravenously (i.v.) injected [135]. Also, after i.v. administration MSN-based DDS was found to be accumulated mainly in liver (73%), kidneys (15%) and spleen (7%) while after inhalation it accumulates only 17%, 9% and 1% in liver, kidneys and spleen respectively [135].

As mentioned previously, drug resistance can be observed if P-gp is overexpressed, because MDR-1 will lead to the formation of efflux pump which will pump out the chemotherapeutic agent [152]. Meng et al. developed MSN as a carrier which could simultaneously deliver siRNA targeting P-gp and DOX to the KB-V1 cervical cancer cells leading to increased intracellular concentration of DOX [79]. The MSN was further coated with PEI which helped in conjugation with siRNA. It was discovered that the simultaneous delivery of siRNA and DOX resulted in increased intracellular concentration of DOX and that DOX could be released from the lysosome by a proton-sensitive mechanism [79].

Meng et al. also further used MSN, functionalized by a polyethyleneimine–polyethylene glycol (PEI-PEG) copolymer to deliver DOX and P-gp targeting siRNA. On i.v. administration of the PEI-PEG coated DOX-siRNA MSN, it was observed that ~8% of the administered particle dose was retained in the tumor site. It was discovered that there was significantly enhanced (80%) tumor inhibition with PEI-PEG coated DOX-siRNA MSN compared to DOX (62%) alone or scrambled siRNA (62%) alone. It was also found that DOX associated systemic side effects; including cardio toxicity was reduced after the co-delivery. There was also a significant P-gp knockdown by siRNA from the MSN at various tumor sites and which was also found to be linked to the regions where DOX was released intracellularly [136].

Calcium phosphate (CaP), the inorganic components of biological hard tissues are biocompatible and are not toxic to the mammalian cells [126]. Li et al. utilized this property of CaP and formulated lipid coated calcium phosphate (LCP) nanoparticles for the efficient delivery of siRNA constructs [153,154]. Li et al. further developed anisamide-targeted LCP nanoparticles to efficiently target sigma receptor-expressing NSCLC and deliver siRNA into the cytoplasm (Fig. 3). In this study, a range of pooled therapeutic siRNAs were chosen [human homologue of mouse double minute 2 (HDM2), c-Myc and VEGF] and investigated for their efficacy in inhibiting A549 and H460 NSCLC. The size and zeta potential of the targeted LCP nanoparticles was found to be around 38.6 ± 3.6 nm and 29.1 ± 1.3 mV, respectively. It was found that LCP nanoparticles did not form aggregates when incubated in 50% v/v serum inferring bio stability of CaP nanoformulations. The effect of targeted pooled siRNA combinations (HDM2/c-Myc/VEGF = 1:1:1) containing LCP nanoparticles was observed on A549 tumor cells and it was found that it inhibited gene expression of HDM2, c-Myc and VEGF, with up to 87.6% silencing observed in case of HDM2. The flow cytometry analysis of this siRNA combination therapeutics revealed that there was a significant increase in apoptosis with the targeted LCP nanoparticle group compared to the non-targeted LCP nanoparticle group.

On i.v. injection into A549 xenograft mice, the targeted pooled siRNA(HDM2/c-Myc/VEGF = 1:1:1) LCP nanoparticles accumulated mainly in the tumor cells, with only moderate levels in other organs such as liver and kidney, demonstrating significantly increased tumor penetration and uptake. On treatment with targeted pooled siRNA LCP nanoparticles, there was a significant reduction in tumor growth in H460 and A549 xenografted mice compared to the non-targeted pooled siRNA LCP nanoparticles. The toxicity assay revealed that pooled siRNA LCP nanoparticle formulation was non-toxic as the levels of secreted liver enzymes Aspartate aminotransferase and alanine amino transferase were all unchanged and also there was no organ damage [48].

To overcome the limitations of vectors to deliver siRNA and pDNA specifically to cytoplasm and nucleus respectively, Canine et al. also designed a novel genetically engineered bio polymeric based platform technology termed as FDNT [155,156]. The originally proposed polymer consisted of a DNA condensing and endosomolytic domain with repeated units of arginine- histidine, a pH-dependent fusogenic peptide to destabilize endosomal membrane, a HER2 targeting antibody and M9 nuclear localization signal (NLS) these.

Same group of investigators further modified the biopolymer to successfully deliver siRNA to cytoplasm and pDNA to cell nucleus [157]. The authors found that FDNT/pEGFP complex was able to successfully deliver pDNA to the nucleus mainly due to the presence of NLS and on the other hand NLS lacking FDT was able to successfully reach cytoplasm and deliver its genetic contents. The nanoparticles formed with FDNT/GFP-siRNA and FDT/GFP-siRNA was found to be around 121 ± 7 and 140 ± 5 nm in size respectively. The cell toxicity assays were used to evaluate the synergistic effects of FDNT/pSR39 complexes plus gancyclovir in combination with FDT/BCL2-siRNA complexes and

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Table 1
Co-delivery of siRNA in combination with chemotherapeutic drug and/or nucleic acid based reagent for the treatment of cancer.

siRNA/miRNA	Drug	Type of nanocarrier	Cell lines	*In vivo* model	targeting	Targeting moiety/peptide	References
siRNA targeting BCL2 and MRP-1	DOX/CIS	Mesoporous silica nanoparticle	A549 human lung adenocarcinoma	Murine A549 lung cancer Orthotopic model	Active	LHRH peptide	[135]
siRNA targeting P-gp	DOX	mesoporous silica nanoparticles	MDR KB-V1 human cervical carcinoma	–	Passive	–	[79]
siRNA targeting P-gp	DOX	PEI-PEG functionalized mesoporous silica nanoparticles	MCF-7/MDR—breast cancer	Murine MCF-7/MDR breast cancer Xenograft model	Passive	–	[136]
siRNA targeting mTERT	PTX	HTCC nanoparticles	LLC—lewis lung carcinoma	–	Passive	–	[137]
siRNA targeting GFP	DOX	G(4)-PAMAM-PEG-DOPE dendrimers	C166 cells—yolk sac endothelial	–	Passive	–	[138]
siRNA targeting Luc gene	DOX	(G3) poly (L-lysine) OAS dendrimer	U-87 glioblastoma	–	Active	RGD peptide	[139]
siRNA targeting BCL-2	Docetaxel	PEG-PLL-PLLeu cationic micelles	–	Murine MCF-7 breast cancer Xenograft model	Passive	–	[81]
siRNA targeting MCL-1 and GL2	SAHA	TLO cationic liposomes	KB epithelial cancer	Murine KB epithelial cancer Xenograft model	Passive	–	[82]
siRNA targeting VEGF	PTX	PDMAEMA–PCL–PDMAEMA cationic micelles	PC-3 human prostate cancer and MDA-MB-435-GFP breast cancer	–	Passive	–	[92].
siRNA targeting VEGF and c-Myc	DOX	Lipid polycation DNA nanoparticles	MDR NCI/ADR-RES ovarian tumor	Murine NCI/ADR-RES ovarian cancer xenograft model	Passive	–	[140]
siRNA targeting c-Myc	DOX	Liposome-polycation-DNA nanoparticles	HT-1080 fibrosarcoma	Murine HT-1080 fibrosarcoma xenograft model	Active	PEGylated NGR (asparagine-glycine-arginine)	[141]
siRNA targeting BCL2 and MRP-1	DOX	DOTAP cationic lipid nanoparticles	MDR lung cancer MDR A2780/AD ovarian cancer	–	Passive	–	[142].
siRNA targeting MCl-1	MEK inhibitor PD032590	Cationic liposomes	KB epithelial cancer	Murine KB epithelial cancer xenograft model	Passive	–	[143]
siRNA targeting VEGFR and EGFR	CIS	PEI complexes	–	Murine A549 NSCLC xenograft model	Passive	–	[93]
siRNA targeting X linked inhibitor of apoptosis	PTX	Deoxycholic acid-PEI complexes	HCT-116 colorectal cancer	Murine HCT-116 xenograft model	Passive	–	[144]
siRNA targeting BCL-2	DOX	Cationic PEI-PCl nanoparticles	C6 Glioma Bel-7402 human hepatoma	Murine C6 glioma xenograft model	Active	Folic acid	[145]
siRNA targeting P-gp	PTX	PLGA-PEI nanoparticles	JC mouse mammary cancer	Murine BALB/c JC breast cancer xenograft model	Active	Biotin	[146]
siRNA targeting MCL-1	PTX	Cationic solid lipid nanoparticles	KB epithelial cancer	Murine KB epithelial cancer xenograft model	Passive	–	[147].
siRNA targeting Plk1	PTX	PEG-b-PCL-b-PPEEA micelleplex	MDA-MB-435 breast cancer	Murine MDA-MB-435 s breast cancer xenograft model	Passive	–	[148].
siRNA targeting BCl-2	S-1	Lipoplexes	DLD-1 colorectal adenocarcinoma	Murine DLD-1 colorectal adenocarcinoma xenograft model	Passive	–	[149].
iMdr-1-shRNA iSurvivin-shRNA	DOX	Poly (b-amino esters) based nanoparticles	MCF-7 human breast adenocarcinoma	Murine BALB/c MDR MCF-7 breast adenocarcinoma xenograft model	Passive	–	[150]
siRNA targeting HMD2,c-Myc	VEGF siRNA	Lipid coated calcium nanoparticles	A549 adenocarcinoma and H460 lung carcinoma	Murine A549 and H460 NSCLC xenograft model	Passive	–	[48]
siRNA targeting c-Myc and MDM2	VEGFR mir-24a	Liposome-polycation-hyaluronic acid	–	Murine B16F10 melanoma xenograft model	Active	scFv	[151]

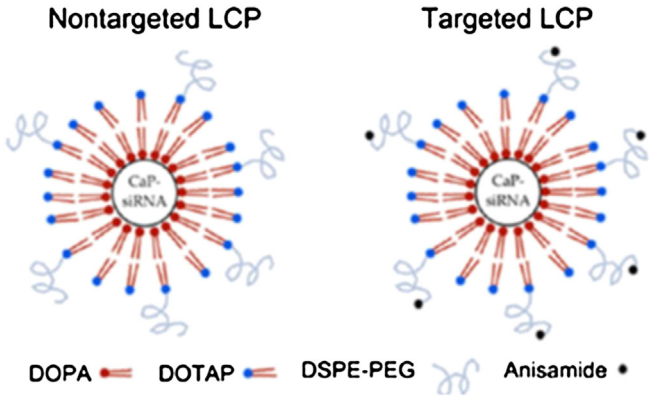

Fig. 3. Schematic representation of non-targeted and targeted LCP nanoparticles adapted with permission from Ref. [48].

observed statistically significant enhanced cell death in SKOV3/GFP breast cancer cells [157]. However, transfection efficiency is relatively lower with inorganic nanoparticles and hence surface functionalized architects continually being suggested to improve their transfection capacity. Further studies are needed to establish this class of nanocarriers for the successful delivery of RNAi combinations.

Despite of progress in the formulation and evaluation of inorganic nanoparticles [158], a standardized and reproducible method is still needed to assess the efficacy and toxicities. In order to develop safer and efficacious nanotechnology based formulations the efficacy and toxicity evaluation of the inorganic nanoparticles is essential. In addition, there is need for systematic studies focused on the pharmacokinetics of the inorganic nanoparticles to evaluate the mechanism underlying toxicities.

5.2. Natural chitosan polymeric nanoparticle based siRNA nanoparticles

Chitosan is a modified natural carbohydrate polymer prepared by the partial N-deacetylation of chitin, a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters [159]. Chitosan nanoparticles have gained more attention as drug delivery carriers because of their stability, low toxicity, simple and mild preparation method [160]. It is found that capacity of chitosan to enhance the absorption and permeation of drugs at GI mucosal sites is compromised due to deprotonation at physiological pH [161]. It has also been found that chitosan gets easily degraded in the lysozyme in the serum [162,163]. Ma Guang-hui et al. developed a partially quaternized derivative of CS *N*-((2-hydroxy-3-trimethylammonium) propyl) chitosan chloride (HTCC) to deliver poorly water soluble drugs by oral route.

Wei et al. used the HTCC nanoparticles (HNP) to deliver siRNA and hydrophobic chemotherapeutic drug paclitaxel (PTX). The prepared siRNA HNPs were found to be in the range of 130–145 nm and found to have colloidal stability. The co-delivery system (HNP/siRNA/PTX) at very low drug concentration (3 nmol/L of siRNA) significantly improved the *in vivo* anticancer activity against lung carcinoma cells and showed no significant side effects. The co-delivery system (HNP/siRNA/PTX) simultaneously delivered the two drugs into the cell which demonstrated the synergistic effects exhibited by the formulation [137]. These are among the few reports on successful application of chitosan based nano-architect to deliver siRNA in combination with other drugs for cancer therapy.

There has been progress achieved in the area of drug delivery using chitosan nanoparticles [164,165]. Although, chitosan has been used to deliver both hydrophillic and hydrophobic therapeutic agents and to formulate multifunctional nanoparticles an investigation focused on evaluation of chitosan based nanoparticles needs to be done. Also, further exploration is warranted for toxicological evaluation considering it's the Generally Regarded As Safe status by US Food and Drug Association (USFDA) for *in vivo* use [166,167].

5.3. Dendrimers based siRNA combinations

Dendrimers, are monodisperse highly branched macromolecules which are discovered in early 1980' by Donald Tomalia and coworkers [168,169]. Dendrimers are monodisperse, nanoscale sizes that matches with the size of biomolecules [170]. Their size and molecular mass is easily controllable and their solubility characteristics can be varied based upon the nature of surface groups [171]. Dendrimer surfaces may be functionally designed to enhance or resist *trans*-cellular, epithelial or vascular permeability [172]. Mathematically defined numbers of terminal surface groups (*Z*) present on dendrimers are suitable for conjugation of drugs, signaling groups and targeting moieties [173]. Dendrimers can also be employed to attain pH reliant release with a slower release under normal physiological conditions and a burst release of loaded bioactive at the acidic tumor environment [173]. Dendrimers are routinely synthesized as tuneable nanostructure that may be designed and regulated as function of their shape, size, surface chemistry and interior void space [203].

Several polyamine polymers have been explored as carriers for siRNA delivery including poly(amido amine) (PAMAM) dendrimers. The PAMAM dendrimers, also known as starburst dendrimers are the first one to be investigated which included ammonia as the core [174]. Cationic dendrimers have been used as non-viral delivery vectors for efficient siRNA delivery [175]. In a similar investigation on dendrimers, Minko et al. developed tumor targeted delivery system using surface-engineered poly (propyleneimine) dendrimers with siRNA caged inside the dendrimers (Fig. 4). PEGylation and caging modification stabilized the system and extended its systemic circulatory lifetime [175].

Recently Kaneshiro et al. prepared symmetric octa (3-aminopropyl) silsesquioxane (OAS) based poly (L-lysine) octasilsesquioxane dendrimers (nanoglobules) having a globular morphology, a rigid structure and a highly functionalized surface. Kaneshiro et al. also used the nanoglobules to form conjugate with large number of Gd (III) chelates to prepare nanoglobular MRI contrast agents [176]. The generation 3 (G3) poly(L-lysine) OAS dendrimer was used to develop Arginylglycylaspartic acid (RGD) targeted nanoglubules for co-delivery of DOX and siRNA targeting firefly luciferase. The DOX was conjugated to the nanoglobular surface via a biodegradable disulfide spacer and further cyclic RGDfK peptide (RGD) was conjugated via a PEG (2000) spacer to yield G3-[PEGRGD]-[DOX] conjugate. SiRNA was further

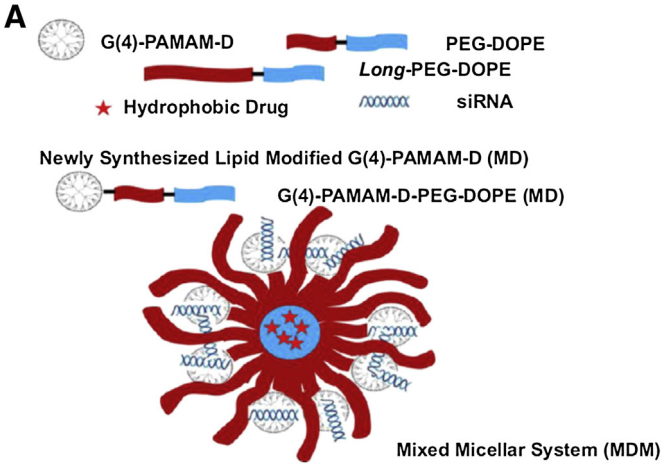

Fig. 4. Schematic illustration of the formation of mixed micellar system using G (4)-PAMAM-D-PEG-DOPE/PEG-DOPE mixed micellar system. A poly (ethylene glycol) - dioleoylphosphatidyl ethanolamine (PEG-DOPE) modified G (4)-PAMAM nanocarrier used to deliver siRNA targeting green fluorescence protein. (Adapted with permission from [138].)

complexed with G3-[PEG-RGD]–[DOX] conjugate to form a targeted co-delivery system. Cytotoxicity studies in U87 glioblastoma cells revealed that targeted G3-[PEGRGD]-[DOX] showed enhanced cytotoxicity than the non-targeted control G3-[DOX] and free DOX.

Fluorescence confocal microscopy in U87 glioblastoma cells revealed that the G3 conjugates were effective in facilitating the intracellular uptake of siRNA. It was observed that targeted conjugates, G3-[PEG-RGD]-[DOX] and G3-[PEG-RGD) resulted in reduced intracellular uptake of siRNA compared to non-targeted G3 nanoglobule and G3-[DOX], which may be due to the interaction of higher positive surface charge on non-targeted G3 nanoglobule and G3-[DOX] with negatively charged cell surface. The targeted nanoglobular drug conjugate G3-[PEGRGD]-[DOX] mediated intracellular gene silencing efficiency of an anti-Luc siRNA was evaluated in U87 glioblastoma cells and it was found that the siRNA complexes of G3-[PEG-RGD]-[DOX] resulted in the enhanced gene silencing efficiency (75%) compared to siRNA G3-[PEG-RGD] (50%), which also attests to the fact that anticancer drug and siRNA can be loaded onto dendrimeric nanoglobules and conjugated with targeting agent for intracellular co-delivery of chemotherapeutics and siRNA [139].

In another study, Biswas et al. modified G(4) PAMAM nanocarrier with poly (ethylene glycol)–dioleoylphosphatidyl ethanolamine (PEG-DOPE) to synthesize a new construct G(4)-PAMAM-PEG-DOPE. This construct was used to deliver siRNA and hydrophobic drug (DOX) to the aveolar adenocarcinoma cells. The siRNA complexed with dendrimers was stable and exhibited complete protection against enzymatic degradation, compared to free siRNA which showed partial instability in 1 h and complete enzymatic digestion within 6 h [138].

Dendrimers represents a versatile nanocarrier for chemists towards fabrication of siRNA/miRNA nanoformulations with amendable terminal structure to attain prolonged circulatory lifetime, sustained release of bioactives and targeting potential [177,178]. Also the dendrimers have a higher loading capacity for the delivery of the drugs into tumor tissues. However, more persuasive as well as comprehensive statistics acknowledging the safety-toxicity issues of dendrimers are primarily warranted to ascertain this nanocarrier as a pragmatic alternative, particularly in the field of cancer therapy.

5.4. Cationic nano micelles based siRNA combinations

Recently, the cationic micelles have been widely explored in the delivery of drugs and RNAi based combinations [92,179]. The cationic micelles are nanoscopic core/shell structures formed by amphiphilic block copolymers [180]. The inherent and modifiable properties of micellar architect makes them well suited for drug delivery applications. The key advantages of nanomicelles include solubilization of poorly water soluble molecules, sustained release, and protection of encapsulated bioactives from degradation and metabolism [181]. Peptide based cationic micelles have been studied lately as gene transfection vectors due to their biocompatibility and biodegradability. Cationic micelles are showing a huge promise when it comes to delivery of various hydrophobic and hydrophilic drug, but faces stability issues which needs to be overcome for it to reach the clinical trials.

Deng et al. synthesized novel cationic micelles, primarily based on hybrid polypeptide copolymers poly(ethylene glycol)-b-poly(L-lysine)-b-poly(L-leucine) (PEG-PLL-PLLeu) to effectively transfect genes [182]. The same group used the cationic micelles to encapsulate negatively charged siRNA (BCL-2) and hydrophobic DTX and investigated the synergistic tumor suppression effect against breast cancer cells and the ability to simultaneously deliver siRNA and DTX.

The siRNA and DTX co-loaded nanoparticles were around 121.3 nm in size and zeta potential was 20.48 mV. A reduction in cell proliferation to 8.9% was observed with siRNA and DTX co-loaded nanoparticles. A synergistic inhibitory effect of the DTX and siRNA combination on tumor growth was demonstrated by siRNA and DTX co-loaded nanoparticles against breast cancer cell. The survival rates of the nude mice receiving siRNA and DTX co-loaded nanoparticles were significantly enhanced compared to the mice receiving PBS, or the two therapeutic agents alone [81].

In another study based on cationic micelles, Shim et al. synthesized oligolysine-based cationic lipid derivatives and encapsulated siRNA (targeting green fluorescence protein) and anticancer drug suberoylanilidehydroxamic acid (SAHA) for co-delivery [82]. The trilysinoyl oleylamide (TLO) based cationic liposomes was mainly made up of DOPE, which served as the lipid component and is also a fusogenic peptide which enhances the cellular delivery of siRNA. The siRNA loaded lipoplexes were found to be in the range of 190–230 nm and zeta potential of 67.2 ± 12.0 mV. The zeta potential of SAHA loaded TLOL (trilysinoyloleylamide liposomes) was 19.7 ± 0.4 mV after complexation with luciferase (siGL2). After treatment of KB cells with siMcl1/pSTLOL (PEGylated SAHA trilysinoyloleylamide liposomes) the non-viable epithelial cancer cells were increased by 2.6–3.4 fold compared to siMcl1/pTLOL and siGL2/pSTLOL treatment respectively. siMCl1/pSTLOL also exhibited significantly enhanced *in vivo* anticancer activity. The combination of siGL2 complexed with pSTLOL and SAHA also showed no lethality or abnormal behavior upon i.v. administration [82].

There have been many reports of use of polydimethylaminoethyl methacrylate (PDMAEMA) for gene delivery mainly due to its relatively low toxicity and high buffer capacity [183,184]. Zhu et al. developed cationic micelles based on PDMAEMA–PCL–PDMAEMA triblock copolymers for the combinatorial delivery of PTX and siRNA (Fig. 5). Reversible addition-fragmentation chain transfer (RAFT) polymerization of dimethylaminoethyl methacrylate (DMAEMA) was used to prepare the PDMAEMA–PCL–PDMAEMA triblock copolymers. The particle sizes of micelles of PDMAEMA–PCL–PDMAEMA triblock copolymers were found to be in the range from 53.6 to 132.2 nm with positive surface charges ranging from +29.3 to +35.5 mV. The PDMAEMA–PCL–PDMAEMA triblock copolymer micelles were less toxic than 25 kDa PEI and also biodegradable, which indicates their reduced long term toxicity. The co-delivery of VEGF siRNA and PTX using PDMAEMA–PCL–PDMAEMA micelles resulted in significantly decreased VEGF expression in human prostate carcinoma PC-3 cells compared to delivery of VEGF siRNA alone [92].

Cheng et al. developed a folate conjugated ternary copolymer, FA–PEG–PEI–PCL, of poly (ethylene glycol) (PEG), PEI, and PCL, which was capable of self-assembling into cationic micelles and co-deliver siRNA targeting Bcl-2 gene in combination with DOX. The copolymer exhibited reduced cytotoxicity and increased siRNA/drug delivery performance. The particle size was found to be around 191 nm and zeta potential was found to be around +6.51 mV. The co-delivery of siRNA targeting Bcl-2 gene and DOX resulted in synergistic effect with enhanced DOX induced apoptosis in SKOV-3 breast cancer cells due to the down regulation of anti-apoptotic Bcl-2 gene by siRNA [185].

Despite the vast literature on successful application of cationic micelles for RNAi based systems deliverance, surprisingly there are only few studies focused systematically on the physicochemical properties of siRNA/miRNA micellar systems [186,187]. Hence, looking towards immense potential and versatility, more systematic approach is warranted to evaluate these nanosystems for delivery of RNAi based combinations. This literature gap also widened the scope of formulation scientists to look for alternative delivery approaches that has more clinical as well as commercial production like “liposomes.”

5.5. Lipid based nanoparticles/liposomes

Liposomes are spherical structures in which the inner aqueous layer is covered by outer lipid bi layers [188]. Liposomes are biocompatible and can be used to deliver both hydrophilic and hydrophobic drug [189]. The periphery of liposomes can be modified to render them long circulatory lifetime and site specific delivery to tumor tissues. Liposomes are especially effective in treating diseases that affect the phagocytes of the immune system because the liposomes tend to accumulate

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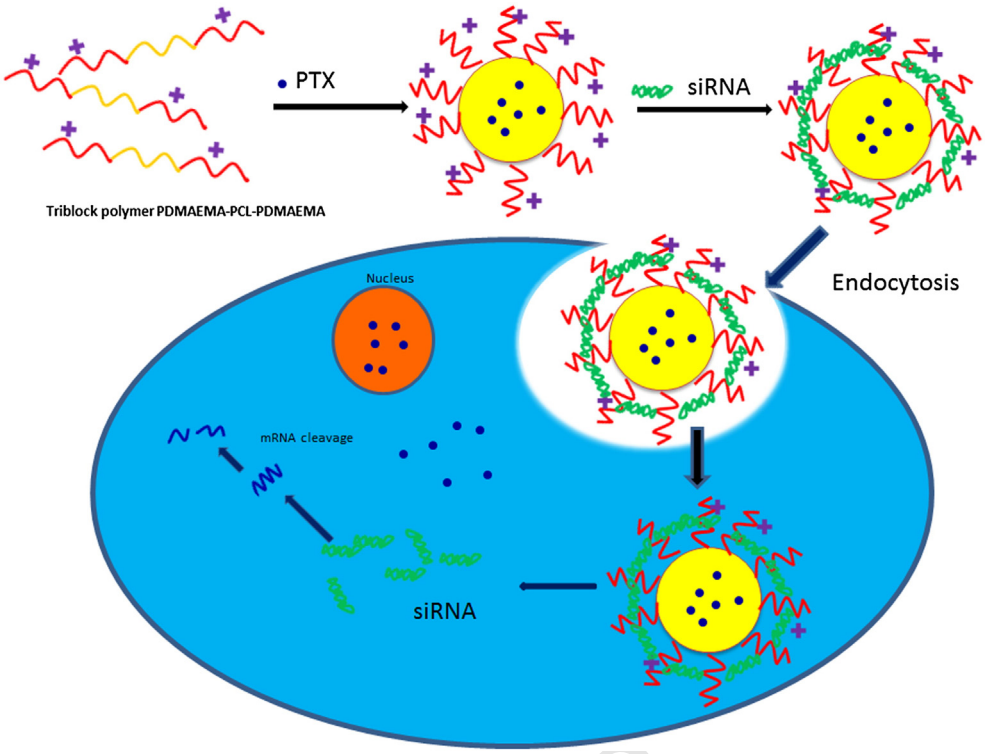

Fig. 5. Schematic representation of self-assembled cationic micelles of PDMAEMA–PCL–PDMAEMA triblock copolymers for the simultaneous combinatorial delivery of PTX and siRNA. The figure depicts the release of siRNA from the cationic micelles inside the cell and degradation of mRNA resulting in its action.

in the phagocytes which recognize them as foreign invaders [190]. Liposomes size, charge and other characteristics can be altered according to the drug and the desired site of action [190]. Liposomes provide a great opportunity to deliver therapeutic agent for cancer therapy and have been widely used for this purpose [189].

5.5.1. Lipid based nanoparticles/liposomes siRNA combinations

Chen et al. developed targeted cationic lipid-polycation-DNA (LPD) nanoparticles, containing PEG, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and tethered with targeting moiety anisamide to encapsulate siRNA [191]. However, cationic lipids have poor entrapment efficiency in encapsulating drugs like doxorubicin. To overcome this problem, the same group developed multifunctional anionic liposome-polycation-DNA (LPD-II) nanoparticles, comprised of anionic lipids to deliver VEGF siRNA and DOX simultaneously into MDR ovarian cells.

The LPD-II nanoparticles were modified with anisamide, which is a ligand of sigma receptor and is overexpressed in ovarian cancer cells. The PEGylated LPD-II nanoparticles were found to be in the range of 20–50 nm with a spherical morphology. The co-delivery of VEGF siRNA and DOX using targeted nanoparticles with guanidium containing cationic lipid (DSAA) was resulted in enhanced growth inhibition of NCI/ADR-RES Adriamycin resistant ovarian tumor, probably due to enhanced DOX uptake. An approach of silencing the MDR expression was used to inhibit the growth of tumor cells. The co-delivery of c-Myc siRNA and DOX resulted in enhanced uptake of DOX into cells, probably by downregulating both c-Myc and MDR expression in NCI/ADR-RES ovarian cancer cells. The c-Myc mRNA and protein expression of the NCI/ADR-RES ovarian cells were also found to be significantly reduced [140].

Chen et al. further developed a core/shell type of nanoparticle formulation, called liposome-polycation-DNA complex (LPD) consisting of cationic liposomes and polycation condensed DNA to deliver plasmid DNA or siRNA to tumor cells *in vivo* [191,192]. The same group further utilized the LPD nanoparticles and modified with PEGylated aspargine–glycine–arginine (NGR) peptide, for targeted co-delivery of c-Myc siRNA and DOX. The c-Myc mRNA levels were significantly reduced after treatment of HT-1080 Fibrosarcoma cells with siRNA containing LPD-PEG-NGR nanoparticles. The Western blot analysis and immunostaining results indicated that LPD-PEG-NGR containing c-Myc siRNA can promote cell death in the tumor cells and the apoptosis effect was targeting peptide dependent. Since it has been found that DOX can easily bind to DNA which is a part of LPD, DOX formed a physical complex with LPD siRNA nanoparticles. After complexation with DOX the average size of the LPD-PEG-NGR DOX nanoparticles was 188 ± 29 nm and the zeta potential was 27.2 ± 1.0 mV. The combination of DOX and siRNA coformulated in LPD-PEG-NGR resulted in significant improvement in tumor growth inhibition compared to free DOX and c-Myc siRNA in LPD-PEG-NGR [141].

In another study Saad et al. developed novel multifunctional cationic liposomal nanoparticles, to deliver DOX and siRNA targeted to MRP1 and BCL2 mRNA. DOTAP based cationic liposomes were prepared using ethanol-injection method and later were used to encapsulate and complex DOX and siRNA respectively. The positively charged DOTAP based DOX:siRNA complexes were found to be around 500 nm with a surface charge of around +4 mV.

The fluorescence studies clearly demonstrated that the cationic liposomes were able to penetrate the cancer cells and deliver DOX and siRNA into the cytoplasm. It was also found that the delivery of two siRNA, BCL-2 and MRP1 by cationic liposomes resulted in significant suppression of targeted mRNA: BCL2 and MRP-1 confirming the effectiveness of the combination delivery. The delivery of combination of DOX and siRNA targeted to BCL2 and MRP1 by liposomes significantly enhanced the apoptosis in MDR human lung cancer cells compared to the level of apoptosis achieved by each component of liposomes when applied separately. The IC_{50} dose of the combination of DOX with both siRNA was found to be 20% of that compared to free DOX and the cytotoxicity was almost 4.1 times enhanced than liposomal DOX [142].

In a study by another group, Suh et al. developed a novel amino acid derived lipid *N*,*N*″dioleylglutamide (DG) and formulated cationic liposomes to deliver siRNA [193]. Kang et al. further formulated cationic

DG-containing liposomes (DGL) for the co-delivery of Mcl1 siRNA and MEK inhibitor PD032590 and investigated *in vitro* and *in vivo* anticancer activity against epithelial cancer cells. The size of siRNA complexes with PD032590 loaded DGL (PDGL) was around 229.5 ± 2.6 nm while the zeta potential was around 16.5 ± 2.0 mV. It was found that the Mcl1 expression and pERK1/2 levels were reduced after the cellular co-delivery of siMcl1 and PD0325901 using PDGL and PD0325901 specifically affected proteins involved in the Raf/MEK/ERK signaling pathway, significantly decreasing the levels of pERK1/2. The *in vivo* effects of the siRNA PDGL complex in KB epithelial cancer cell bearing mice revealed that Mcl1 levels and pERK1/2 levels were significantly decreased by siMcl1 and MEK inhibitor PD0325901. The treatment of mice with siMcl1 complexed with PDGL resulted in significant decrease in tumor size by 79% compared to control group [143].

Although PEI complexes conjugated with PEG have shown good transfection as well as silencing effect in combination with siRNA, it often induces severe toxicities to cells through necrosis or apoptosis [194]. Hence, there is a need to develop alternative cationic polymers which exhibit minimal or lack of cytotoxicities and able to efficiently deliver siRNA and chemotherapeutic agents. Kim et al. developed a cationic solid lipid nanparticle (cSLN) system to deliver siRNA (VEGF and GFP) [195]. Same group utilized 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine-based cSLN to deliver PTX and human MCL1-specific siRNA (siMCL1) (Fig. 6). The PTX loaded nanoparticles (PTX-SLN) had average particle size about 140.4 ± 12.9 nm while on complexation with siRNA the size increased to 183.1 ± 12.0 nm. The MCL1 mRNA levels were significantly reduced on delivery of siMCL1 using PTX-SLN and also the survival of cancer cells was found to be lowest. The intratumoral co delivery of PTX and siMCL1 using PTX-SLN resulted in increased inhibition of epithelial tumor growth [147] .

5.5.2. Lipid based nanoparticles/liposomes based miRNA combinations

MiRNA therapeutics development represents a new and promising strategy for the treatment of cancer [120]. Only limited studies have been published on the nanoparticle mediated delivery of miRNA in recent past [151,196]. The lipid based miRNA combination delivery for the treatment of cancer is summarized below.

Chen et al. developed liposome-polycation-hyaluronic acid (LPH) nanoparticle formulation modified with GC4 (phage identified internalizing) single-chain variable fragment (scFv) that target tumor sphere cells, a tumor-targeting human monoclonal antibody for systemic delivery of siRNA and miRNA into experimental lung metastasis of murine B16F10 melanoma model. The size and zeta potential of the siRNA and miRNA encapsulated LPH nanoparticles were around 170 nm and 10.9 ± 4.8 mV. The targeted nanoparticles showed efficient cytosolic delivery of the fluorescein isothiocyanate (FITC) labeled siRNA in B16F10 tumor cells. The protein expression of c-Myc, MDM2, and VEGFR was suppressed in the B16F10 lung metastasis, after the combined delivery of siRNA with GC4 targeted nanoparticles, indicating simultaneous silencing by siRNAs.

It was discovered that the growth of the metastasis nodules was suppressed after the combined siRNA delivery by the GC4 targeted nanoparticles and also the tumor load decreased to 30%. The combination of siRNAs and miR-24a delivery by GC4 targeted nanoparticles additively inhibited tumor growth as the tumor load decreased to about 20% compared to 30% and 50% when treated with siRNAs and miR-34a alone. MiR-34a down regulates the surviving expression in the lung metastatic tumor. The PEGylated siRNA and miRNA GC4 targeted nanoparticles showed minimal or no toxicity as the pro-inflammatory markers [interleukin (IL)-6, IL-12, and interferon (IFN)-γ] were not induced and the hepatotoxicity marker (aspartate aminotransferase and alanine aminotransferase) levels were same in the C57BL/6 mice [151].

These studies briefing the delivery of miRNA combinations for cancer therapy indicated the use of lipid based nanocarrier. However, detailed investigation pertaining to its physical, biophysical and storage stability is urgently warranted to evaluate the use of lipid based nanoconstructs for delivery of miRNA based combinations. The investigations to determine the toxicity should be performed with special emphasis on long term exposure toxicities in animals, and humans to optimize existing technologies for clinical use [197].

5.6. Polyethyleneimines co-blocks based siRNA combinations

Positively charged cationic polymers have been widely studied as vectors to efficiently deliver gene to the cancer cells [198]. PEI is one such cationic polymer that has been extensively studied as non-viral vector for efficient gene delivery [199,200]. It has been proven that PEI is responsible for the proton sponge effect inside the endosome resulting in rupturing of the endosomal membrane and helping DNA/siRNA–PEI complex to release [201,202]. The major disadvantage with PEI is its cytotoxicity, which has been to some extent eliminated by coating with human serum albumin [203] and PEGylation [204,205].

Boussif et al. explored the use of PEI for siRNA delivery and found that the positively charged PEI-siRNA complex protected the siRNA from degradation *in vivo* and facilitated subsequent siRNA release from endosomes due to proton sponge effect, after uptake by cellular endocytosis mechanisms [206]. Chen et al. used the PEI complexes to formulate PEI-siRNA (VEGFR2 and EGFR) complexes and evaluated *in vivo* antitumor effects in combination with CIS in murine A549 NSCLC tumor xenograft models. The combination of VEGFR2 siRNA + EGFR siRNA + CIS was resulted in significant downregulation of VEGFR2 and EGFR mRNA levels compared to siRNAs administered individually [93].

Chae et al. proposed a novel polymeric conjugate system comprising of a molecular amphiphile (bile acid) and a cationic polymer PEI to mediate gene transfection [207]. The increased transfection, which

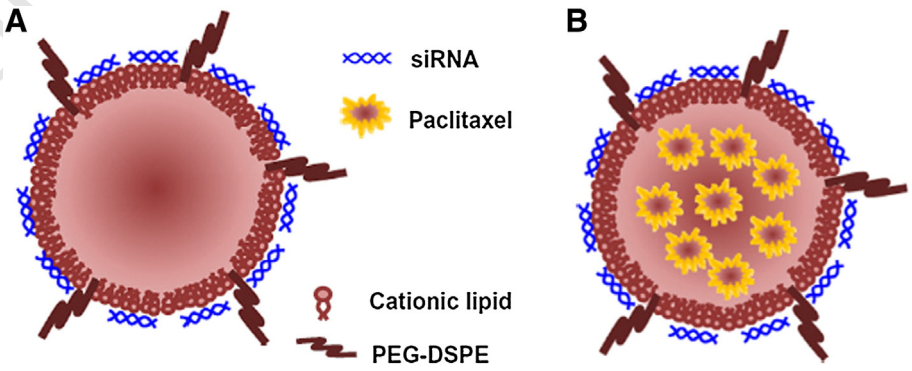

Fig. 6. Schematic representation of cationic solid lipid nanoparticles complexed with siRNA. A) Empty solid lipid nanoparticles. B) PTX loaded solid lipid nanoparticles (adapted with permission from [147]).

occurred via membrane translocation of the polyplex particles was independent of endocytosis and energy. Same group utilized the micelle forming property of the conjugate for the co-delivery of PTX and siRNA targeting X linked inhibitor of apoptosis (XIAP) gene [144]. The deoxycholic acid-PEI, DA3 of around 88.4 nm with spherical morphology was used as a platform for the co-delivery of siRNA and drugs. The combination of PTX and DA3 siRNA demonstrated an enhanced cytotoxic effect on the HCT-116 colorectal cancer cells with around 71% reduction in cancer cell viability compared to 54% and 45% observed with PTX/DA3 and DA3/siRNA combination respectively. The intratumoral injection of the combined formulation (PTX/DA3/siRNA) demonstrated a significantly enhanced inhibitory effect on tumor growth and also completely impeded the tumor growth [144].

In another study, Cheng et al. developed a novel diblock copolymer of PCL and linear PEI (PEI-PCL) and assembled into biodegradable cationic nanoparticles to encapsulate BCL-2 siRNA and DOX. The PEI-PCL nanoparticles were further coated folic acid–polyethylene glycol and poly (glutamic acid) (FA-PEG-PGA) on the surface of cationic PEI-PCL nanoparticles to target folate receptor in C6 glioma cells and impart stability to the multifunctional nanoparticles (Fig. 7) [208]. The multifunctional nano-assembly co-loaded with siRNA and DOX was about 184 nm in size and having a positive surface charge of +5.1 mV. The nano-assembly was also found to be stable in serum, showed preferable drug release profile and increased transfection efficiency in human hepatoma Bel-7402 cell lines. The folate-targeted multifunctional nano-assembly simultaneously delivered siRNA and DOX into C6 cells resuting in a synergistic effect. At 24 h post injection of DOX-PCE/BCL-2/FA showed increased fluorescences of DOX and siRNA in tumor tissue sections from rats compared to adjacent normal tissue. The folate targeted co-delivery of DOX and siRNA resulted in significant tumor growth inhibition compared to non-targeted formulations [145].

Recently, Huang et al. developed polymeric micelles based on PEI-stearic acid (SA) grafted polymer. The PEI-SA micelle provides with the advantage of incorporating hydrophilic moieties in hydrophilic shell while the hydrophobic drugs can be incorporated in the hydrophobic core. The co-loading of anti-VEGF siRNA and DOX in the micelles resulted in the significant reduction in the hepatoma growth. The siRNA binding efficiency was significantly increased with the PEI-SA micelles compared to PEI alone. SiRNA delivered with the micelles exhibited improved stability and cellular uptake efficiency compared to the free siRNA [209].

5.7. Polymeric nanoparticles based siRNA combinations

Polymeric nanoparticles have unique physicochemical properties such as ultra-small and controllable size, larger surface area to mass ratio, and functionalizable structure [210]. The polymeric nanoparticles have been shown to alter and improve the pharmacokinetic and pharmacodynamic properties of various bioactive molecules. The above mentioned properties of polymeric nanoparticles can be applied to overcome some of the limitations in traditional drug delivery approaches [211]. Polymeric nanoparticles have been used *in vivo* to protect the drug in the systemic circulation, and to deliver the drug at a controlled rate to the site of action while minimizes undesirable side effects [212]. Following section mainly describes various polymer based nanoparticles used to co-deliver siRNA and chemotherapeutic agents.

PLGA nanoparticles have been proved to be biocompatible and non-toxic in several studies [213,214]. In another study, Patil and Panyam found that PLGA nanoparticles alone resulted in poor encapsulation of siRNA and thus introduced PEI in the polymer matrix to successfully increase the siRNA encapsulation [215]. Same group further used targeted PLGA-PEI nanoparticles to encapsulate siRNA targeting P-gp and PTX functionalized with biotin to target breast cancer cells. Scanning electron microscopy studies and dynamic light scattering studies showed that PTX-siRNA nanoparticles were spherical in shape with average particle size of about 228 ± 22 nm respectively. The biotin functionalized PTX-siRNA nanoparticles were having a negative surface charge (−12.1 ± 0.3 mV). The co-delivery of siRNA and PTX using nanoparticles improved cytotoxicity in drug resistant JC breast tumor cell line compared to nanoparticles containing PTX alone. The combination treatment with PTX-siRNA nanoparticles resulted in significant increase in PTX accumulation in JC tumor cell lines. On *i.v.* injection of the biotin conjugated dual agent nanoparticles in mice bearing tumors, a significant tumor growth inhibition was observed, compared to the non-targeted dual agent nanoparticles [146].

Sun et al. developed an amphiphilic biodegradable triblock copolymer poly (ethylene glycol)-b-poly (ε-caprolactone)- b-poly (2-aminoethyl ethylene phosphate) PEG-b-PCL-b-PPEEA based system called as "micelleplex." The triblock polymer having the ability to self-assemble and form micellar nanoparticles, with hydrophobic core comprised of PCL and PPEEA and PEG as cationic shell and hydrophilic corona respectively (Fig. 8). The negatively charged siRNA and hydrophobic PTX was encapsulated in the micellar nanoparticles to form a "two-in-one" micelleplex. The cellular uptake studies using rhodamine (Rho) and fluorescein (FAM) labeled PTX and siRNA, respectively; demonstrated micelleplex delivered the drug and siRNA into the cells simultaneously. SiRNA targeting polo-like kinase 1 (Plk1) packaged micelles (micelleplexsiPlk1) demonstrated dose dependent knockdown of the expression of target gene Plk1, at 62.5 nM and 125 nM which led to 32% and 78% knockdown respectively. Also simultaneous delivery of siPlk1 and PTX by PTXmicelleplexsiPlk1 demonstrated synergistic inhibition of the proliferation of MDA-MB-435s cancer cells. PTXMicelleplexsiPlk1 was able to increase cell apoptosis to ~58% with formulations containing 0.005 μg/ml PTX and 125 nM siPlk1 compared to ~16% with siPLK loaded Micelleplex siPlk1 [148].

In recent years, a novel oral fluoropyrimidine derivative, designated S-1, consisting of three pharmacological agents Tegafur (TF), 5-chloro-2, 4-dihydroxypyrimidine, and potassium oxonate in a molar ratio of 1:0.4:1, has been studied extensively for its effectiveness in treating various cancers [216]. However it showed a limited anticancer activity as a single agent mainly due to the ability of cancer cells to evade apoptosis.

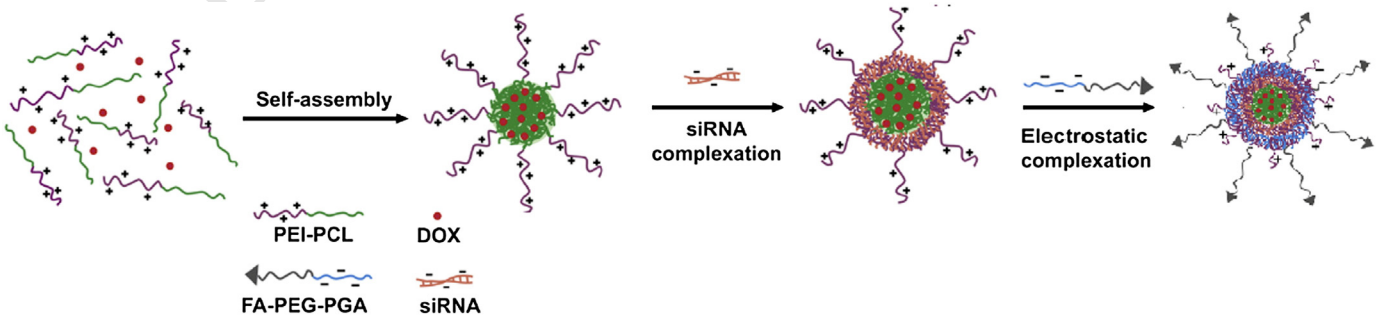

Fig. 7. Schematic illustration of the formation of multifunctional nanoassemblies comprising of DOX and siRNA. (Adapted with permission from [208].)

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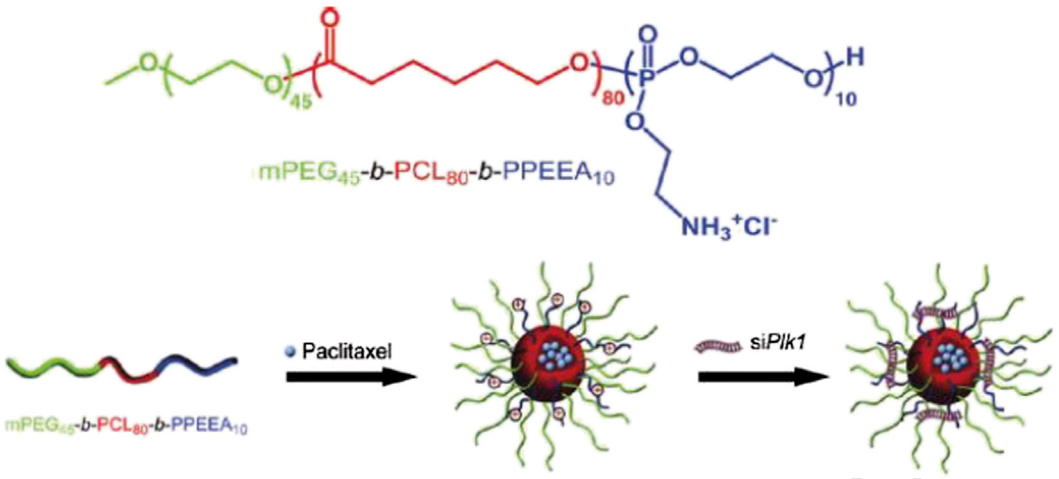

Fig. 8. A schematic illustration of the formation of micellar nanoparticles (adapted with permission from [148]).

To overcome this problem, Nakamura et al. used S-1 in combination with siRNA targeting Bcl-2 (antiapoptotic protein). The SiRNA was encapsulated in PEG coated lipoplexes and on simultaneous administration with S-1 induced significant breast tumor growth suppression [149].

Poly (b-amino esters) (PAEs) are biodegradable and have been used as vehicles to deliver RNA [217,218]. In order to improve its gene delivery efficiency Yin et al. prepared disulfide bond containing PAE, poly [bis(2-hydroxylethyl)-disulfide-diacrylate-b-tetraethylenepentamine] (PAP). The intracellular reductive glutathione and thioredoxin will result in cleavage of the disulfide bond and release the contents. The effect of combination of PAEs-based RNAi and DOX was investigated on mice xenograft model bearing MDR lung cancer. The combination of chemotherapy DOX and two RNA (iMdr-1-shRNA and iSurvivin-shRNA) was resulted in a synergistic effect on overcoming MDR [150].

The complexity of polymeric nanoparticles as multicomponent three dimensional structures requires careful designing and engineering [219]. To achieve reproducible formulations it is also important that scale-up and manufacturing processes are systematically studied [220] . The safety and efficacy of the nanoparticles has to be carefully examined in various preclinical and clinical studies as it can be easily influenced by change in the nanoparticle properties [219].

5.8. Polymerosomes based siRNA combinations

Polymersomes are the polymeric vesicles that undergo self-assembly in hydrophillic solutions from block copolymers and have been widely studied as potential drug delivery candidate since last one decade [221,222]. The polymersomes were able to conjugate biologically active ligands, such as avidin, antibodies and biotin, to their surface and, thus, provide targeted therapy and imaging strategy [223] . It was reported that polymersome could be used in controlled release of multiple drugs due to its EPR effect and relatively higher drug loadings into polymersome compared with liposomal formulation. Polymersome encapsulating DOX and/or PTX was widely researched as a treatment for cancer. Overall, polymersomes have great delivery potential owing to their advantages, such as robust and larger shell enhancing drug loading and stability, and possibility of enhanced drug targeting and prolonged circulatory lifetime [224].

Past work has highlighted peptide-functionalized polymersomes as a highly promising targeted delivery system. Polymersomes seem to possess most of the mandatory attributes required for successful siRNA/miRNA delivery. Its aqueous core allows successful loading of hydrophilic nucleotides sequences, while their release can be effectively controlled through either oxidation-sensitive or hydrolysis-sensitive block copolymer amphiphiles [225]. Polymersomes were reported to be circulating *in vivo* for much longer than lipid vesicles and cationic carriers [226]. In addition, copolymer degradation can generate surfactants that promote endolysosomal release as already exploited in the nuclear delivery of a DNA-intercalating drug [227].

In an early report, Pangburn investigated co-encapsulation and delivery of siRNA inside peptide-functionalized polymersomes composed of poly (1,2-butadiene)-b-poly (ethylene oxide) (PRb). The authors primarily concluded PRb peptide-functionalized polymer vesicles to be a promising system for siRNA (targeting Orai3 gene) delivery to specifically attain cell kill in T47D breast cancer cells, while preserving viability of noncancerous MCF10A breast cancer cells. Reports are also available that support polymersomes to be primarily releasing their payload in the early endosomal and successful escape from endosomes to cytosolic compartments. These report suggested a promising first generation replica for targeted delivery of siRNA [228].

Kim et al. described oligonucleotides and siRNA (targeting Lamin A/C protein) co-loaded polymersomes and demonstrated their efficient delivery into A549 lung adenocarcinoma cells. Fluorescent-oligos and fluorescent-copolymer were utilized for visualizing the cellular uptake and nuclear delivery of cargo. The authors demonstrated the efficient knockdown of the lamin protein in cultured cancer cells with oligo/siRNA loaded polymerosomes with selective nuclear localization and cell specific expression activity in mdx mouse model. It was inferred that the surfactant generated by the degradation of the carrier provides a means of escape of the payload from the confining endolysosomal compartment and facilitates the desired spatial relocalization of released oligonucleotide to the nucleus as well as functionally active siRNA in the cytosol [222].

Kim et al. also reported that combination therapy via co-delivery of siRNAs and an anticancer drug (DOX) can be a promising strategy due to the synergistic effect [225]. In this study, Bcl-xL siRNA and DOX are encapsulated into designed methoxy-PEG-block-poly(D,L-lactic acid) (mPEG-b-PLA) block copolymer polymersomes. Cytotoxicity evaluation of Bcl-xL siRNA and DOX co-encapsulated polymerosomes (CPsomes) showed enhanced inhibition of cell growth and apoptosis in MKN-45 and MKN-28 human gastric cancer cell lines than that of siRNA alone and DOX loaded formulation. These results demonstrated that co-delivery of siRNA and chemodrugs using polymerosomes results in synergistic activity and indicates the potential of polymerosomes as efficient nanocarriers for siRNA based combination therapy [225].

The *in vivo* toxicity of delivery r systems has always been of crucial apprehension. Previous studies with polymersome indicate a maximum tolerated dose that exceeds 35 mg/ml after systemic injection and no measurable cytotoxicity to C2C12 and BAEC endothelial cell lines [229]. It is also imperative to make a note that in *in vivo* studies with polymerosomes containing siRNA-DOX, the final concentration of

copolymer injected into *mdx* mice was comparatively low (at1 mg/ml), and increased doses needs to further evaluated [225].

The configuring capability of architect and properties of polymersome has considerably projected these nanoarchitects for delivery of RNAi based combinations. Further, the aptitude to polymersome to get tailored for targeting chemistries makes them an ideal platform for the encapsulation of a broad range of therapeutic molecules with RNAis based therapeutics (like dyes, nucleic acids, proteins). Further, it will also be an interesting area of research to comparatively assess the delivery attributes of long worm-like micelles with polymerosomes.

The main goal of delivery of siRNA/miRNA/drugs using a nanocarrier is to protect the therapeutic agents against degradation and also to deliver them at the target site i.e. tumor cells. In addition, the use of nanocarriers should also have reduced toxicity while maintaining the therapeutic effects of therapeutic agents and should allow ease of attachment of a targeting ligand [230]. However, none of the nanocarriers mentioned above fulfil all the criteria mentioned above [230]. Some nanocarriers such as dendrimers and liposomes facilitate incorporation of hydrophobic and hydrophilic agents while face the problem of low biodegradation and drug leakage respectively. Polymeric micelles on the other hand allow incorporation of hydrophobic therapeutic agents but the toxicity of degradation products needs to be considered. The inorganic nanoparticles such as silica are easy to fabricate and functionalize while there is a lack of data on their long term toxicity. The translation application of these nanoparticles with defined dosing regimen for the treatment of cancer evaluated under preclinical setup is lagging. A number of factors such as, difficulty in synthesizing the nanocarriers in large quantities for clinical trials along with the regulatory obstacles warrant further investigations to translate the nanocarriers from bench to bedside [231]. With the progress made in nanotechnology combined with polymer chemistry one can hope for a solution to overcome these hurdles. Meanwhile we have to follow the strategy of "Horses for courses," where depending upon the target and the therapeutic agent a specific nanocarrier can be selected and used for the treatment of cancer.

6. Ongoing clinical trials on RNAi based combinations: Current status

Silenseed Ltd. is conducting a Phase II study with a siRNA drug in combination with chemotherapy to treat advanced pancreatic cancer (Table 2). The National Cancer Institute reports that the disease accounted for 38,460 deaths in 2013 with 45,220 new cases reported and is responsible for 6% of cancer deaths each year. The study involved administration of chemotherapy (gemcitabine) and single dose of siG12D LODER in which siRNA targeting mutated-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) oncogene KRASG12D (siG12D) was encompassed in a small biodegradable polymeric matrix. Upon administration, siGD12 inhibited transcription of KRAS proteins and resulting in reduction in the pancreatic tumor growth. KRAS is found to be associated with tumor cell proliferation and reduced survival and is also found to be mutated in over 90% of human pancreatic ductal adenocarcinomas (PDAC) [232].

Another Phase I study reported for the treatment of pancreatic cancer involving administration of PEGylated liposomal siRNA in combination with CIS [232]. SiRNA targeting ERCC1 was selected as excision repair cross-complementation group 1 (ERCC1), which is involved in DNA repair mechanism leading to CIS resistance. The increased expression of ERCC1 results in removal of CIS-induced bulky adducts from the cancer cells. The inhibition of transcription of ERCC1 mRNA by the siRNA will help reduce or eliminate the CIS resistance and lead to CIS-induced apoptosis and ultimately reduction in tumor size.

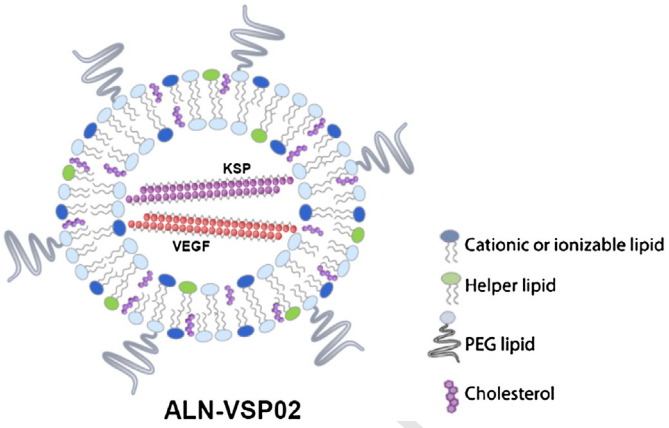

Fig. 9. Lipid nanoparticle for systemic delivery (adapted from online alnylam pharmaceuticals).

Alnylam Pharmaceuticals in partnership with Tekmira developed a lipid nanoparticle carrier system encapsulating two siRNAs to target mRNA of vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) mRNA (Fig. 9). It is the first dual targeted RNAi drug, which targets two pathways with two different siRNAs, thus increasing the potential therapeutic effect. Stable nucleic acid particle (SNALP) carrier encapsulating the two siRNAs and is passively targeted against liver cancer [233]. Preliminary pharmacodynamics data suggest ALN-VSP02 was able to show anti-VEGF effect in majority of treated patients and when administered i.v. was well tolerated in most of the 28 initial patients [232]. The progress of RNAi combinations from lab to clinical settings requires efficacy and safety evaluation under preclinical trials. Further, in coming years more RNAi based combination based formulations are anticipated to enter in clinical trials with successful transformations of the products in commercial markets.

7. Conclusion and future directions

The co-delivery of siRNA/miRNA with chemotherapeutic agents provides promising option to overcome chemo resistance. Clear evidences are given by the recent reports that combination delivery of siRNA/miRNA and drug using nanoparticles are indeed helpful in inhibiting the tumor growth compared to siRNA, miRNA or drug alone. Various nanocarriers have been developed to deliver siRNA and drug; however these nanocarriers are also not devoid of limitations. The ideal nanocarrier system should protect the drug and RNAi therapeutic agent from the circulatory environment and efficiently deliver the therapeutic agents to tumor cells. There is also a need to study the safety profiles of the various carriers used in the *in vivo* delivery of these therapeutic agents with special focus on their toxicity and immune response. SiRNA/miRNA can play first line role in the combination drug

Table 2
Clinical trials for siRNA based combinations.

Targeting	Company	DDS	Drug	Indications	Status	Reference
Passive	Silenseed Ltd.	Biodegradable capsule containing siRNA + Chemotherapy	siG12D LODER + Gemcitabine	Advanced pancreatic cancer	Phase II	[232]
Passive	Silenseed Ltd.	PEGylated liposomal siRNA + chemotherapy	siRNA (targeting ERCC1) + Cisplatin	Pancreatic cancer cells	Phase I	[232]
Passive	Alnylam	Lipid based nanoparticle carrier system	SiRNA (VEGF) + siRNA (kSP)	Liver cancer and metastatic liver disease	Phase I	[233]

delivery system. In a combination therapy including various nucleic acid base reagents, siRNA/miRNA play the primary role in inhibiting the growth of tumor cells by targeting various genes which are involved in the tumor growth, progression and or survival. While in combination with drug, siRNA/miRNA can play a secondary role in which it can target various genes which are involved in developing chemo resistance and thus overcoming or reducing the drug resistance in tumor cells thereby enhancing the anticancer activity.

The earlier reports of the clinical trials of the combination delivery consisting of siRNA/miRNA and anticancer agents are very promising, however there are few number of nanoparticle systems based on siRNA/miRNA have been approved by FDA. There are several obstacles in the clinical development of RNAi-based therapeutics. The major challenges for RNAi-based therapeutics include minimizing the potential off-target effects related to the sequence of both dsRNA strands and controlling the specificity of the siRNA. The pharmacokinetic and pharmacodynamic issues have also not been well defined in most of the studies related with the *in vivo* siRNA delivery. The siRNA/miRNA target cell machinery that is common to both normal and tumor cells, thus there is also a need to develop targeted delivery systems to overcome the associated side effects. Furthermore, there are financial risks for the pharmaceutical companies as the delivery of these RNAi based agents are challenging and the cost of manufacturing and scale up of products are potentially higher. It also has to be taken into account that an alteration of multiple genes, mutations of proteins, and associated downstream cascade are involved in the pathogenesis of cancers. To deliver effective therapeutic concentrations of RNAi using targeted nanocarriers to the tumor cells, a dose adjustment studies also have to be performed.

It is anticipated that the research on combination delivery of RNAi therapeutic agents and chemotherapeutic drugs will progress with increase in the knowledge and innovative delivery strategies. With continuous development the combination delivery system will ultimately lead toward availability of effective therapies for cancer. Despite advancement of siRNA based combination therapies to Phase II and Phase III trials, there are limitations associated with siRNA combination delivery. The clinical trials of siRNA based combinations for the cancer therapy has shown how far these approaches are used, although there are many hurdles needs to be overcome for using the novel delivery technologies.

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