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REVIEW

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Nanoparticulate RNA delivery systems in cancer

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Abstract

Background: Drug delivery system is a common practice in cancer treatment. RNA interference-mediated post-transcriptional gene silencing holds promise as an approach to knockdown in the expression of target genes responsible for cancer cell growth and metastasis. RNA interference (RNAi) can be achieved by delivering small interfering RNA (siRNA) and short hairpin RNA (shRNA) to target cells. Since neither interfering RNAs can be delivered in naked form due to poor stability, an efficient delivery system is required that protects, guides, and delivers the siRNA and shRNA to target cells as part of cancer therapy (chemotherapy).

Recent findings: In this review, a discussion is presented about the different types of drug delivery system used to deliver siRNA and shRNA, together with an overview of the potential benefits associated with this sophisticated biomolecular therapy. Improved understanding of the different approaches used in nanoparticle (NP) fabrication, along with an enhanced appreciation of the biochemical properties of siRNA/shRNA, will assist in developing improved drug delivery strategies in basic and clinical research.

Conclusion: These novel delivery techniques are able to solve the problems that form an inevitable part of delivering genes in more efficient manner and as part of more effective treatment protocols. The present review concludes that the nanoparticulate RNA delivery system has great possibility for cancer treatment along with several other proposed methods. Several NPs or nanocarriers are already in use, but the methods proposed here could fulfill the missing gap in cancer research. It is the future technology, which unravels the mystery of resolving genomic diseases that is, especially genomic instability and its signaling cascades.

KEYWORDS

drug delivery, metastasis, Ran-GTP, RNAi, shRNA, siRNA

1 | INTRODUCTION

The promising drug release characteristics of nanodimensional delivery systems make them a potent tool in gene therapy, where the genetic characteristics of the patient can be targeted specifically during cancer treatment.[1-4] This concept may be possible by using suitable biotherapeutics, such as siRNA that can be encapsulated in a drug delivery vehicle and administrated to the target site without compromising their ability to deliver drug at the site of target.[4-6] Originally the viral method of delivery was used but due to its limitations

like the use of viruses in production on a large scale, toxicity and immunogenicity, the method is not preferred and a need for a new method is essential[7] (Table 1).[6,8-10] Different types of nanoparticulate delivery systems, like lipid-based vesicles, ketals nucleoside lipid nanoparticles (NPs), cell penetrating peptides NPs, polymersomes, chitosan-based NPs have been utilized by researchers to deliver siRNA for cancer treatment.[4,5,11-15] The process by which the target gene expression is reduced by siRNA is known as RNA interference (or RNAi). RNAi is a powerful process, which can be used to knock-down abnormal gene expression, especially in many cancers where the perturbed gene expression can lead to the growth and metastasis of cancer cells. For example, there is accumulating evidence that the eukaryotic protein Ran (also known as Ran GTPase) is overexpressed in a number of different cancers. Ran overexpression leads to the migration of cells from the primary tumor to a secondary site, where they colonize and form another tumor in a process called metastasis.[16-18] Furthermore, Ran silencing has been shown to induce apoptosis in tumor cells, whereas Ran depletion has minimal effect in normal cells.[19] It is hypothesized that the administration of a specific siRNA encapsulated within a nanodimensional delivering system could silence *RAN* overexpression and it may also be possible to induce apoptosis in the target cell.[14] In addition to siRNA, similar effects have been observed when small hairpin RNA (shRNA) has been utilized to target genetic aberrations in cancer cells.[20,21] Like siRNA, shRNA can also deplete the target gene expression via RNAi, but an efficient non-viral gene delivery system is required for improved administration. In this review, a discussion will be presented on the possible methods of fabricating novel drug delivery systems, which could encapsulate siRNA/shRNA, and be used as anticancer or antimetastatic biotherapeutics.

2 | NANOPARTICULATE DELIVERY SYSTEMS

The ability of NP to therapeutic agents, like siRNA or shRNA for the treatment of genetic disease, such as lung cancer, breast cancer, and Alzheimer's disease makes them a potent tool for gene therapy.[22-24] Their ability to mimic biological membranes and evade the immune system makes them ideal vehicles for drug delivery. The fabrication of a nanoparticulate delivery system, which can be utilized for more specific delivery of drugs has always been a priority for improved disease treatment. Nanodimensional delivery systems came into existence in the late 1960s,[25,26] when researchers exploited the self-assembling properties of block copolymers[26] and manufactured drug carrying, hollow vehicles. Self-assembly is a thermodynamically driven and reversible phenomenon, where molecules form a well-defined structure that is held together by weak, noncovalent forces. Self-assembling properties of various block copolymers (or surfactants) have been exploited and used successfully to manufacture a biocompatible and biodegradable drug delivering vehicles.[27-30] Moreover, various shapes of NP can be attained by adjusting the packaging criteria of the self-assembling molecules (or surfactants), (Figure 1). In addition to manufacturing NP, it is also possible to design their shape according to the specific requirements. The purpose of the nanodimensional drug vehicles is to unload the encapsulated drug to the target site, which will further help in the treatment of disease. It is evident that many researchers have manufactured different drug delivery systems and successfully delivered the drug at the point of target, for instance, polyglycerol-based dendrimers were produced and used by Paula Ofek et al against human glioblastoma and murine mammary adenocarcinoma cells.[31-35] The main challenge in drug delivery is to overcome the physiochemical and biological barriers such as the cell membrane, cell environment, blood serum protein, endosomal pathway, and immune system to allow successful drug administration.[36] Moreover, the biodegradable and biocompatible properties are equally important for the development of an effective NP. To achieve a NP associated with all the aforementioned properties, researchers have used different formulations of molecules or surfactants to create NP via various innovative methods.

2.1 | Polymersomes

Polymersomes are the spherical hollow bodies, tailored to respond to the desired stimulus and used to deliver both hydrophilic and hydrophobic drugs.[37,38] For instance, diblock copolymer of poly(ethylene glycol) (PEG) and an acid-labile polycarbonate, poly(2,4,6-trimethoxybenzylidenepentaerythritol carbonate)-based polymersomes were used to encapsulated hydrophobic paclitaxel and hydrophilic

TABLE 1 Viral vs nonviral drug delivery

S. No	Parameters	Viral delivery system	Nonviral delivery system	References
1	Immunogenicity	Causes induction of inflammatory system that leads to degeneration of transducted tissues	Comparatively very less transduction effect to tissues and immune system	8
2	Transgenic capacity size	Limited transgenic capacity for example 38 kb in adenoviruses	No limitation in size of transgenic DNA	9
3	Toxicity	High toxic in nature	Low cytotoxicity	6,10
4	Example	Retroviral vectors	Poly(lactic-co-glycolic acid) (PLGA) nanoparticles	6,10
5	Applications	Used for familial hyperlipidemia gene therapy and tumor vaccination	Used for delivery of antitumor drugs and applicable in many therapeutics	8,10

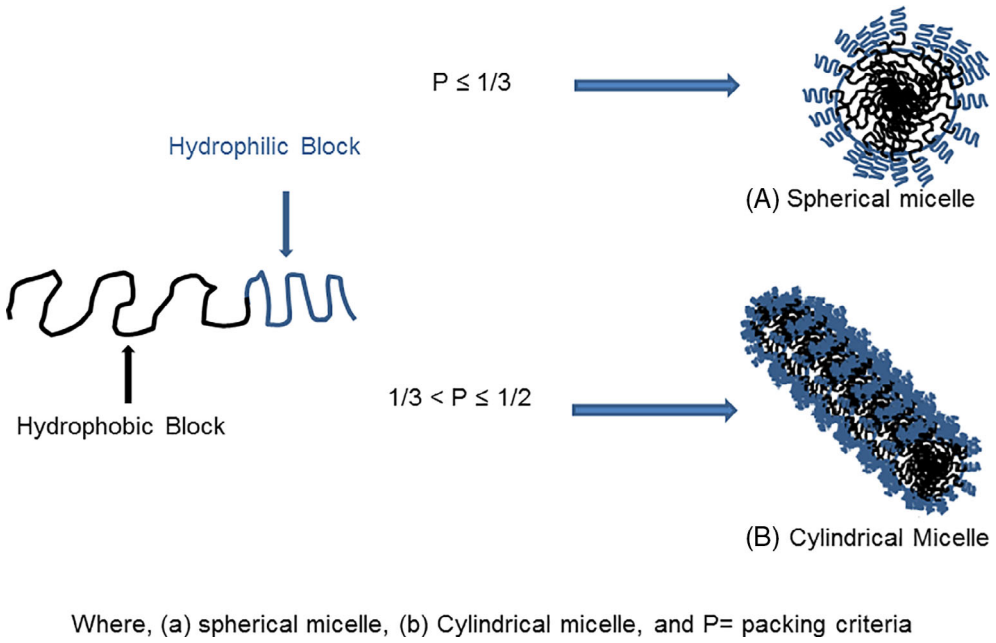

FIGURE 1 Self-assembly of polymers, different geometries formed by coblock polymers, A, spherical micelle formed at $P \leq 1/3$, B, cylindrical micelle when $1/2 \geq P > 1/3$. Where "*P*" is the packing criteria

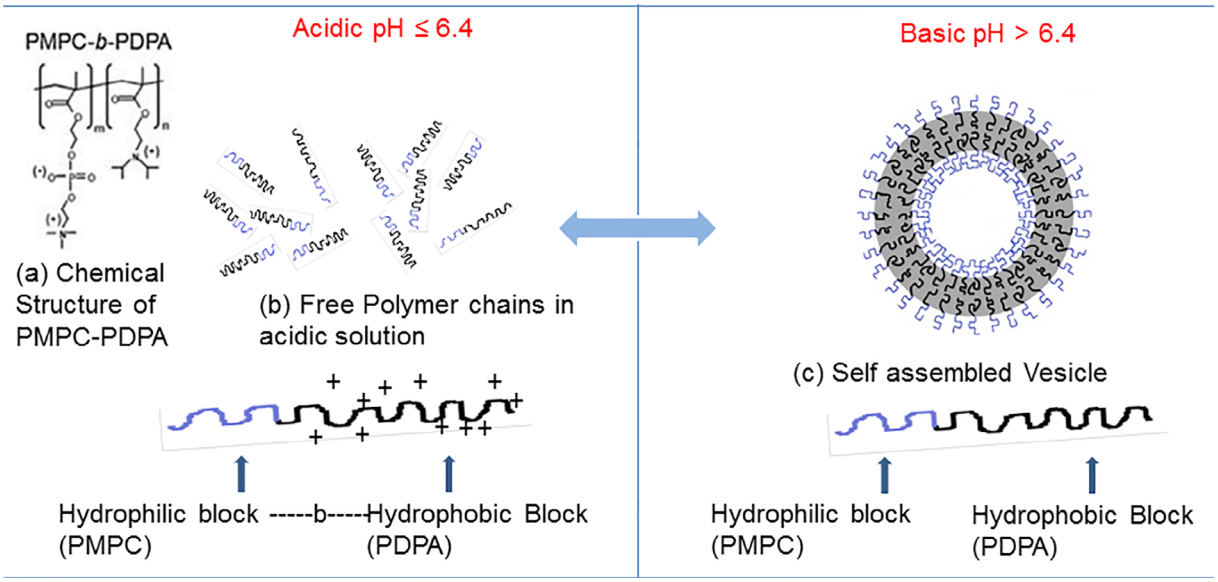

FIGURE 2 Self-assembly of PMPC-PDPA based polymers into vesicles where at acidic pH, that is, pH < 6.4 polymers remains in the form of free chain, whereas on pH > 6.4 they forms vesicles. A, Chemical structure of PMPC-PDPA, B, free polymer chain in acidic solution, and C, self-assembled vesicle—(adapted and modified from Massignani et al).[42] PMPC-PDPA, poly (2-methylacryloxyethyle phosphoryl choline)-block-(2, diisopropylamino ethylemethylacryate

doxorubicin hydrochloride simultaneously.[39,40] Moreover, Battaglia et al manufactured biocompatible and biodegradable amphiphilic diblock (poly (2-methylacryloxyethyle phosphoryl choline)-block-(2, diisopropylamino ethylemethylacryate) or PMPC-PDPA derived copolymeric hollow pH-sensitive nanovesicles (called polymersomes) via a thin film rehydration method for intracellular drug delivery.[38,41] In this method, the polymer solution was mixed with chloroform/methanol solution and left in a vacuum to produce a thin film of polymer. This thin film was subsequently rehydrated with aqueous NaOH to generate the polymersomes. At a low pH, the tertiary amine group of PDPA block becomes protonated and the hydrophobic blocks become more hydrophilic, resulting in free polymeric chains in solution. These chains form vesicles upon making a basic polymer chain solution (Figure 2).[42] The advantage of using pH-sensitive PMPC-PDPA driven polymersomes is that they can be used to encapsulate both hydrophilic and hydrophobic drugs depending on the method of fabrication.[31] Moreover, siRNA incorporated within the PMPC-PDPA-based polymersomes have been delivered within the oral squamous cell

carcinoma cells cell line SCC-4 successfully.[43] Unlike liposomes or niosomes that have leaky structures, polymersomes seem resilient and stable.[32-34,44] The thick corona of polymersomes gives them a rigid structure, which makes them more resilient as they circulate in blood plasma for prolonged time periods. The only drawback faced when using polymersomes is the slow content release due to the vigorous and a stable bilayer membrane.[45] To overcome this disadvantages stimuli-responsive polymersomes are used extensively.[46]

2.2 | PEG-based micelles

The surface properties of NP play a significant role in dictating their intracellular fate. As the plasma membrane is anionic in nature, NPs are required to be positively charged to allow them to cross the cell membrane barrier.[47] The electrostatic interaction between the negatively charged plasma membrane and the cationic NP would allow the NP to cross the plasma membrane barrier and thus NP enters into the cell. Apart from this, the cationic nature of NP enables them to encapsulate the negatively charged phosphate backbone bearing siRNA with the help of electrostatic interactions. In contrast, excess charge from the NP may cause a problem with the delivery, as the presence of excessive charge may not only create toxicity, but could also cause NP to bind with the negatively charged serum proteins present in plasma, hence affecting delivery properties.[47] To solve the problem of negatively charged serum proteins binding with cationic NP, PEG has been conjugate to the surface of NP, which could prevent particle aggregation in the presence of serum proteins and provide steric stabilization.[34] Scientists have exploited the good pharmacokinetic properties of PEG with fabricated NP to encapsulate siRNA and have used them as antimetastatic biotherapeutics.[15] A novel class of PEG-based trilayer pH responsive micelles were synthesized by dissolving poly (ethylene glycol)-block-poly(N-(N[2-aminoethyle]-2AMinoethyle) aspartamide)-block-poly(e-caprolactone) or PEG-b-PAsp(DET)-b-PCL in acetone and distilled water with vigorous stirring followed by centrifugation.[15] Bichambered, trilayered hollow vehicles were formed which could be used to load both hydrophilic and hydrophobic drugs simultaneously. Researchers have been using PEG-based micelles successfully to treat malignancy and claim it to be one of the most promising nanocarriers.[48]

2.3 | Lipoplexes

Another class of nanovehicles, called lipoplexes, are a combination of cationic lipids with nucleic acids. Lipoplexes may be used in drug delivery but their high level of toxicity and low endosomal escape are a significant concern.[49] To address this, lipoplexes have been coated with PEG in an attempt to solve this challenge.[49] Poly(ethyleneimine) (PEI)-based drug delivery systems may also be observed in siRNA delivery to cancer cells where an siRNA and PEI complex is formed by mixing siRNA (in 150 mM NaCl) and PEI (in NaCl) at different concentrations to manufacture siRNA containing nanovehicles for lung cancer treatment.[24] The electrostatic binding of PEI with siRNA has been found to be one of the reasons why siRNA/PEI complexes can be formed successfully. For example, cyclin-B1 siRNA was incorporated with PEI for delivery in a mammary adenocarcinoma model (TSA-luc cells).[24,50,51] In contrast, 1,2-dioleoyl-3-trimethyl ammonium propane (DOTAP) lipoplexes have been used to deliver siRNA against TNF-α transcript to restore immunological balance in rheumatoid arthritis, but little effect was observed.[49] FRα-targeted lipoplexes with therapeutic gene expression regulated by an hTERT promoter might be a promising agent for gene therapy and for successfully treating the ovarian cancer.[52]

2.4 | Chitosan-based nanoparticles

Another biodegradable class of nanovehicles is chitosan-based NP, which have been used successfully to deliver siRNA/shRNA. Chitosan-coated polyisohexylcyanoacrylate NP loaded with RhoA-siRNA has been shown to inhibit significantly mammary tumor growth.[49,53] Moreover, another novel method was used to fabricate chitosan-based NP called ionic gelation method where modified ionic gelation of polyanionic tripolyphosphate with cationic chitosan were engaged together to produce NP for siRNA/shRNA delivery.[54,55] Chitosan/siRNA complexes have been used to deliver siRNA against tumors. For example, chitosan complexes with VEGF-A-siRNA were shown to generate inhibition of metastasis in breast tumor.[56] The chitosan-based drug delivery system has its own benefits yet the reason it is not the most suitable delivery system is due to the poor stability of colloids, which seriously limit the efficiency of drug delivery.[52] This method of delivery is open for further advancements to improve the efficiency of drug utilization, especially for size dependent applications like gene transfer or delivery of drug via mucosal routes.[57]

2.5 | Lipid/calcium/phosphate-based nanoparticles

It has been reported that siRNA (biodrug) loaded along with a phosphorylated drug (chemical drug) caused a high level of apoptosis in cancer cells on delivery and showed almost no toxicity.[4] LCP (lipid/calcium/phosphate) NP were used and also modified with an anisamide, a ligand-specific for recognizing sigma receptors overexpressed in many human cancer cells, for specific delivery.[4] LCP NP showed a long circulation time, specific target, and evasion from the reticuloendothelial system. These NPs were synthesized from water-in-oil microemulsions, where siRNA in the oil phase containing cyclohexane was mixed with Na_2HPO_4 (in cyclohexane) to form a first microemulsion. Simultaneously, a second microemulsion was prepared by mixing $CaCl_2$ and cyclohexane. The two microemulsions were mixed together with cholesterol, chloroform, DOTAP (1.2-dioleoyl-3-trimethylammonium-propane chloride salt), DSPE-PEG (1,2-distearoryl-sn-glycero-3-phosphoethanolamine-N-[methyl-oxy9polyethylene glycol ammonium salt] and DSPE-PEG-AA (PEG-

anisamide) to fabricate LCP NP. The advantage of these NP was their monodisperse nature, which may prevent drug loss and allow the production of appropriate sizes of NP for intracellular delivery. It may be possible to synthesize a nanoparticulate delivery system, with almost no polydispersity, so any loss of drug may be reduced and a more effective delivery system can be obtained.[4] LPD (lipid phosphate DNA) is also commonly used vector for drug delivery the advantage of using LPD is the prolonged circulation time and an elevated endosome release mechanism. If the advantage of LPD and CaP (calcium phosphate) NP could be combined, it would be a great upgrade. Many investigators are frequently trying to prepare nanosized CaP particles to ameliorate the transfection reproducibility and efficiency.[58]

2.6 | Miscellaneous nanoparticles

Different fabrication methods have been adopted to produce drug-carrying vehicles for intracellular delivery. For instance, vesicle formation formulation procedure is reported where DLin-MC3-DMA, distearoylphosphatidyl choline, cholesterol, and PEG-DMG were mixed in a molar ratio of 50:10:38.5:1.5.[59] These NP could be used to encapsulate biodrugs and chemical drugs simultaneously for their delivery within the cell. Microspheres have also been used to deliver siRNA to target cells and one of the most effective methods for their fabrication is the water drying method where water/oil/water emulsions are prepared to form NP. This method was used to prepare microspheres where water/oil emulsion (polyethylene alcohol and dichloromethane) and polyvinyl alcohol were mixed together and stirred to evaporate an organic solvent. Poly(lactic-co-glycolic acid)-based microspheres were obtained from this, which demonstrates a significant amount of naked siRNA encapsulation (ie, 49%). In contrast, encapsulation efficiency (EE) increased to 64% and 80% respectively when a carrier in the form of arginine and polyethylimine in inner water phase during fabrication of microspheres, was introduced.[60] Another newly emerging NP delivery system is the gold NPs, which are promising agents for cancer therapy for instance, PEG-RGD-siRNA bearing gold NP targets LA-4 cancer cells through αvβ integrin interaction, which successfully helped in down regulation of *c-Myc* oncogene expression in alveolar epithelial type-2 adenocarcinoma L4 cells.[61] In another case, nanomicelles composed of P85-PEI/D-α-tocopheryl polyethylene glycol 1000 succinate (or TPGS complex) were manufactured for shRNA delivery, which showed a great resilience and an extended circulation time of more than 24 hours.[62] The method adopted for their fabrication was thin film rehydration where P88-PEI and TPGS were mixed in acetonitrile contained with paclitaxel (PTX). The solvent was evaporated at 40°C and solid PTX/copolymer matrix was obtained, which was then hydrated with distilled water to form rigid nanomicelles. These NP carrying shRNA was used against 4 T1 breast cancer cells and high cellular uptake was observed.[62]

Dendrimers gained considerable attention for their potential applications as drug carrier specifically for nucleic acids.[63,64] Dendrimers are spherical, symmetrical in shape and hyperbranched macromolecules. The special feature of the dendrimers NP is the chemical homogeneity, which allows repeated attachment of the chemical group thereby, it has numerous ligand attachments on the surface making dendrimers as a potential candidate for drug delivery carrier and other biomedical applications. The dendritic polymers have exhibited a high efficacy in the successful deliver of siRNA and knockdown and silencing of luciferase gene overexpressed in murine mammary adenocarcinoma cells.[65] There have been several modifications in dendrimer polymers such as polypropyleneime dendrimers that are attractive nonviral vector for siRNA delivery.[66] Dendrimer-mediated delivery of siRNA is a significant and promising approach but it is not yet fully developed due to several limitations including majorly the nonspecific cytotoxicity, which is seen in higher generation dendrimers.[65]

Further, gold/silver NPs are used for delivering siRNA to the targeted gene for a successful knockdown and silencing of the overexpressed genes. Gene silencing of EGFP in CHO-K1 cells expressing EGFP after the addition of siRNA/PEI-AuNPs has been reported.[67] Similarly, silver nanoparticles (AgNPs) with Quercetin (Qe) were fabricated. The modified Ag-NPs-Qe enhanced the antibacterial activity of AgNPs. The Ag-NPs-Qe was further stabilized with siRNA, which showed that the siRNA/Ag-NPs-Qe could be a potential nanoscale drug delivery system for *Bacillus subtilis* targeting bacteremia.[68]

Carbon nanotubes (CNT) are NPs that emerged as a new and an alternate approach to the current tools for delivering therapeutic agents. CNT have bioactive peptides, proteins, nucleic acids, and drugs that are translocated and transported to targeted cells and organs. The CNT are single-walled in which siRNA is coiled and it is a novel approach for siRNA cellular delivery Single wall carbon nanotubes. The CNT-siRNA deliver showed the transfection efficiency of 95% with nonspecific toxicity in neuronal cells and cardiomyocytes.[69] The CNT-siRNA also showed a similar result in nonmetastatic human hepatocellular carcinoma cell line (SKHp1). The *in vitro* applications of the CNT-siRNA delivery system showed no significant side effects and also demonstrated a high efficiency.[69] Functional CNT's display low toxicity and hold a great potential in the nanobiomedicines and nanobiotechnology with their nonimmunogenic abilities.

3 | SMALL INTERFERING RNA (siRNA) AND ITS DELIVERY

siRNA is a 20 to 25 base pairs long oligonucleotide used to knockout the expression of target mRNA by annealing to its complimentary sequence via RNAi (Figure 3).[70,71] The extensive use of siRNA as a drug to treat cancer has been attempted and refined since its ability to target mRNA sequences was reported.[72-74]

Initially, it was believed that double-stranded RNA (dsRNA) makes alterations in the target mRNA to reduce its expression. However, it was later discovered that dsRNA gives rise to siRNA with the help of a bidentate enzyme called dicer, which is a member of the RNAse 3 nuclease family.[75] Dicer splits dsRNA into siRNA. Thereafter, siRNA combines with the RISC-AGO2 (RNA-induced silencing complex-Argonaute) complex, causing the release of the sense strand of siRNA

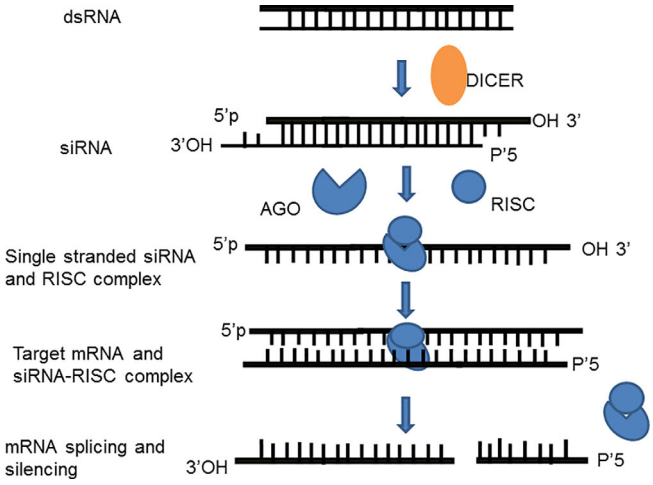

FIGURE 3 siRNA knockdown activity of target mRNA via RNA interference pathway (adapted and modified from Kim et al)[71]

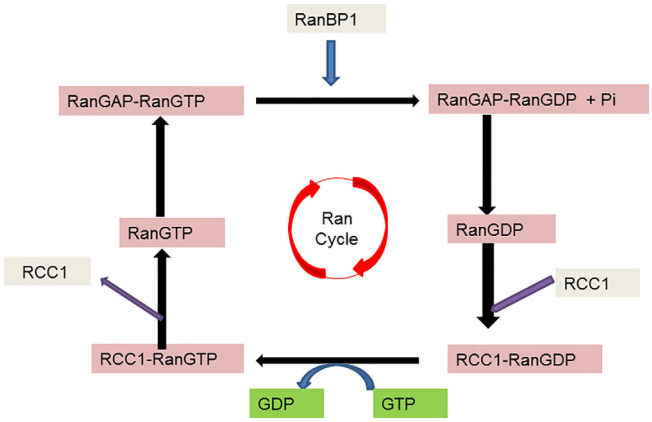

FIGURE 4 Diagrammatical representation of Ran cycle (adapted and modified from Nakielny et al)[79]

but remains linked with its antisense strand. The antisense strand of siRNA and RISC-AGO2 complex attach to the sense strand of target mRNA and cleaves the target mRNA to reduce its expression. The RISC-AGO complex plays a major role in carrying the siRNA to the target mRNA. The AGO component of this complex has three domains, namely the PAZ domain (or Piwi Argonaute Zwille), MID domain (middle domain), and PIWI domain (PIWI protein). SiRNA binds to the MID and PIWI domains from its 5′ end, and the 3′ termini of the siRNA binds to the domain of the AGO complex. When the attached siRNA moves toward the target mRNA, the attachment occurs from the 5′ end up to the 3′ end, which brings the conformational change in the mRNA, causing the PAZ domain to drift away from the 3'end. This leads to the slicing of mRNA and when the cleaved product is released, the PAZ domains returns to its original position at the 3′ end.[76] The phosphodiester backbone of the target mRNA is subsequently broken down by divalent ions (Mg^{2+}).[33]

RNAi ability of siRNA can be utilized to target the cellular pathways of cancer cells that are responsible for cancer growth or metastasis. It has been observed in many tumors that overexpression of Ran GTPase (Ran) leads to uncontrolled growth and proliferation of the cancer cells.[18,77,78] Ran is a eukaryotic nuclear protein that has important function in cell viability, nucleoplasmic transportation, spindle organization, and nucleus formation.[18] It is hypothesized that if Ran protein over expression could be reduced by siRNA-mediated targeting of the *RAN* gene, metastasis of the primary tumor may be prevented.[12] In the Ran cycle, Ran GTP is joined with the Ran GTPase activating protein, RanGAP, which activates RanGTPase leading to the hydrolysis of RanGTP to RanGDP. RanGDP is then converted back to RanGTP by the activity of a nucleotide exchange factor, called RCC1. Moreover, analysis of the Ran cycle (Figure 4),[79] a biological pathway through which Ran is synthesized, shows that RCC1(nucleotide exchange factors) is one of the most important component in the Ran cycle.[80] Therefore, if the action of the nucleotide exchange factors could be inhibited, the possibility of reducing growth and metastasis of cancer cells may be increased.

The process of RNAi was first reported in petunia petals in 1990 and was coined after the discovery where an injection of dsDNA into the *nematode Caenorhabditis elegans* led to a specific silencing of genes highly homologous in sequence to the delivered dsRNA.[34,81,82] RNAi could occur at both transcriptional and post-transcriptional level in plants unlike animals, where it can only occur at the post-transcriptional stage.[83] The property of siRNA that enables it to interfere with the target mRNA sequence and inhibit growth and migration of cancer cells makes it a very useful biodrug in the field of cancer treatment. Since the effect of siRNA is transient, multiple administrations are required and it has been observed that several administrations of siRNA have achieved long term silencing of the target gene without disrupting the endogenous microRNA pathway.[33,83] Another advantage of using siRNA is its zero interaction with the DNA or protein of the target and hence no adverse gene alteration or protein production occurs.[33,83]

3.1 | siRNA delivery to cancer cells

The administration of siRNA directly into the living organisms is not recommended because siRNA is highly unstable and may cause the immune system to react against it and eventually destroy it. In addition, the small size of siRNA could be eliminated from the body by the liver. Another factor, which stops researchers from administrating naked siRNA is its negative charge, due to the presence of an anionic phosphate backbone, which can induce toxicity in the body. Niu et al injected naked siRNA directly into a subcutaneous murine cervical cancer model but multiple administrations were required for any therapeutic responses to be achieved.[84] Moreover, the negative charge of siRNA prevents it from permeating through the plasma membrane barrier, resulting in little, if any, intracellular delivery. Therefore, due to these barriers, siRNA cannot be delivered naked and a delivery vehicle is required that can protect it from immune surveillance and allow it to be delivered inside the cell. Different delivery systems have been used to deliver siRNA within the tumor cells as antimetastatic biotherapeutics. siRNA-EGFR (siRNA-epidermal growth factor

receptor), which promotes differentiation, growth, and metastasis of cancer cells by transmitting growth inducing signals was complexed with PEI and injected into mice bearing lung cancer xenografts; this significantly reduced tumor growth.[85,86] Modified forms of siRNA can be used as alternative method against tumor. For example, the incorporation of 2-o-methyl residues into the sugar structure of selected nucleotides with both sense and antisense strands of siRNA stops activation of the immune response against siRNA.[87] Furthermore, the modification of siRNA with cholesterol may prevent siRNA from exposure to endonuclease activity, which is a barrier in siRNA delivery.[87] On delivery of PAR-1 (protease-activated receptor-1, an angiogenesis inhibitor) siRNA encapsulated within the uncharged DOPC ([1,2-dioleoyl-sn-glycero-3-phosphatidylcholine])-based NP, which proves to be less toxic and shows more tumor cell uptake as compared to cationic liposomes, showed a significant decrease in melanoma growth and metastasis by decreasing angiogenesis.[88,89] PAR-1 regulates gap junction protein connexin-43 and tumor suppressor gene *MAPSIN* to promote metastasis in cancer cells. It has been observed that successful inhibition of TNF-α has been achieved by delivering siRNA in DOTAP (lipoplex)-based NPs.[49] siRNA delivery has also been employed in the case of lung cancer, where chitosan-based NP coated with cyanoacrylate have been used for its delivery to inhibit tumor growth and inhibition.[53] In the case of lung cancer, intranasal, intratracheal, or epithelial delivery can be attempted; however, airway delivery is complicated due to the presence of physiological and immunological barriers. Apart from this, gold NPs have been used to deliver siRNA in human cells, *in vitro* in *Hydra vulgaris* and *in vivo* in nude mice.[90] The target-specific delivery was achieved with the help of RGD (arginine-glycine-aspartate) presented on the surface of the gold NP which is recognized by the cell surface integrin receptors. RGD, also referred to as "molecular glue," may be very helpful in targeting against tumor angiogenesis and metastasis initiated by specific genetic aberrations.[90] PEG-RGD-siRNA bearing gold NPs have been used to target LA-4 cancer cells, through an αvβ integrin interaction, which successfully helped in the down regulation of *c-Myc* oncogene expression in alveolar epithelial type-2 adenocarcinoma L4 cells.[90] αvβ integrin interaction seems to be responsible for promoting tumor angiogenesis.[91] Also, a nanovector modified with RGD may also be helpful in targeting lung and breast cancer. According to a study carried out by Potti et al it is reported that HER2 is expressed in approximately 56% of breast tumors (ductal carcinoma in situ) and 29.5% of lung tumors.[92,93] The immune-liposomes nanovectors with an antitransferrin antibody were used to deliver siRNA to knockdown the gene responsible for HER-2 overexpression successfully.[4] HER-2 promotes cell growth, survival, and angiogenesis by activating many signaling pathways such as the mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and protein kinase C (PKC).[94] This represents another interesting example of target-specific delivery in tumor inhibition in lung or breast cancer cells. Furthermore, a PEI-based nanodelivery system has been observed which was used to deliver siRNA and inhibited survivin and cyclin B1 in lung tumor cells harboring the overexpression of these proteins.[24] Survivin plays an important role in apoptosis inhibition. In addition to this, survivin also plays a critical role in the regulation of cell division by inducing exit from G1 checkpoint arrest and subsequent entry into S phase.[95] It may therefore be possible to induce apoptosis in cancer cells by inhibiting the pathways responsible for it. The two main survival pathways namely, Phosphatidylinositol-3kinase (PI3K)/AkT/mammalian target of rapamycin (mTOR) and Raf/mitogen-activated and extracellular signal-regulated kinase (MEK)/extracellular signal regulated kinase (ERK) or RES-MEK-ERK and PI3K/Akt/mTORC1 (of which Ran is a main target that overexpressed) have been observed which causes metastasis in cancer cells.[18] It has been shown that the aforementioned pathways may coexist in breast cancer cells[93] and inhibition of one pathway may activate the other pathway.[92] These are two of the main signaling pathways that become dysfunctional and hyperactivated in breast, lung, and most of human cancers. In the PI3K/AkT/mTOR pathway, the heterodimer PI3K is activated by G-protein-coupled receptors and the receptor kinase PI3K activates the oncogene AkT. mTOR is composed of two protein complexes known as mTORC1 and mTORC2.[58,62] The mTORC1 pathway can be inhibited by rapamycin or it can also be blocked by using allosteric inhibitors, resulting in the effective treatment of cancer cell lines with PI3KCA mutations transcriptional factors.[96] Both RAS/MEK//ERK and PI3k/AkT/mTOR pathways are hyperactivated in most cancer cells, especially breast and lung cancers,[93,96,97] which can be blocked to induce apoptosis. It was formulated that RNAi could well be employed to suppress these pathways for antimetastasis effects.[98] Another transcriptional factor called c-Myc, responsible for promoting cell growth, proliferation, invasion, and angiogenesis was found overexpressed in nonsmall cell lung cancer cells, which account for up to 85% of all cases of lung cancers.[4] LCP-based NPs have been successfully used to inhibit c-Myc transcriptional factor.[4] The inactivation of the c-Myc pathway could also be a solution to inhibit the metastatic effect of cancer cells. In another study, Zhang et al demonstrated at LCP-based NP fabricated through the double emulsion method could be used to deliver siRNA, resulting in a very high amount of EE, that is, up to 75% EE.[3] Therefore, if we could use the double emulsion fabrication method and use the nanocarriers to deliver and target siRNA against nonsmall lung cancer cells, we could possibly expect improved results. In another case, LCP-based NP were used to deliver siRNA along with the chemical drug gemcitabine monophosphate and the delivery produced a significant induction of tumor cell apoptosis, and reduction in tumor cell proliferation without inducing any noticeable amount of toxicity.[59] Therefore, it is possible to coadminister siRNA with chemical drug which may enhance the quality of treatment in tumor cells. A significant amount of EE was achieved in one case where siRNA microspheres were made by using the water/oil emulsion method and the EE was found to be 48.6 vol%, which is a significant amount for better delivery.[60] It seems that EE is highly influenced by the method of preparation and the type of polymer used for creating the nanovehicles. Another factor, which may account in the EE of NP, is its charging properties. For example, in the case of negatively charged siRNA, the presence of a positive charge on a NP may facilitate siRNA entrapment greatly. The maximum amount of siRNA, which can be encapsulated in a nanovehicle without

disrupting the immune response or causing toxicity needs to be determined for it can change the course of cancer treatment and is still for further advancements and research. Further, the ongoing clinical trials using encapsulated siRNA as a drug against cancer strengthens the anticancer potential of siRNA (Table 2).[99,100]

4 | SHORT HAIRPIN RNA AND ITS DELIVERY

Another potential tool for knocking down the expression of the target gene could be short hairpin RNA or shRNA. It is a small oligonucleotide sequence having a hairpin resembling loop, which could be used as an antimetastatic agent to control secondary growth of cancer.

Several reports have demonstrated that shRNA has been used effectively to knockdown the expression of target mRNA sequences by RNA interference in cancer cells (Figures 5 and 6).[18,101-103] shRNA has proven to be a potential tool against cancer-associated perturbations in gene expression because of its ability to reduce the target gene expression through post-transcriptional gene silencing process.[6] The shRNA formation process starts in the nucleus when the primary transcript is generated by the RNA polymerase II or III promoter. It is then processed into pre-shRNA, which comes into close proximity with a class II RNAase enzyme known as Drosha and DGCR8 complex and as a result, the small shRNA is processed. This complex anneals with small shRNA is then exported into the cytoplasm from the nucleus by exportin 5 with the help of RNA-GTPase dependent mechanism. Then, the small shRNA comes in contact with enzyme called

TABLE 2 siRNA-loaded nanoparticulate drugs in clinical trials

siRNA target	Cancer type	Phase	Nanoparticles	References
EphA2	Advanced cancers	Phase 1	Liposomes	99
Atu027	Advanced solid tumor	Phase 1	Lipid nanoparticles	99
Anti-*KRASG12D* siRNA	Pancreatic ductal adenocarcinoma pancreatic cancer	Phase 1	Lipid nanoparticles	99,100
DCR-MYC	Hepatocellular carcinoma	Phase 2	Lipid nanoparticles	99
KRAS	Ductal adenocarcinoma	Phase 1	LOADER polymeric nanoparticles	100
eIF5A	Multiple myeloma	Phase 1/2	Polyethyleneimine	100
CALAA-001/M2 subunit of ribonuclease reductase	Solid tumor	Phase 1	Cyclodextrin contained polymer	99

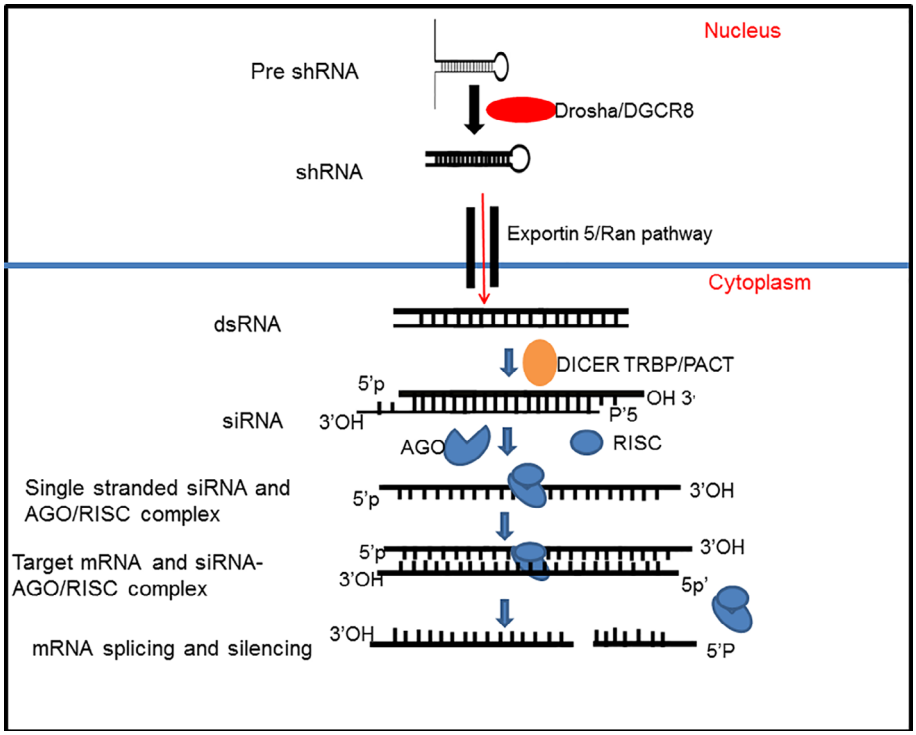

FIGURE 5 shRNA based RNA interference of target mRNA structure

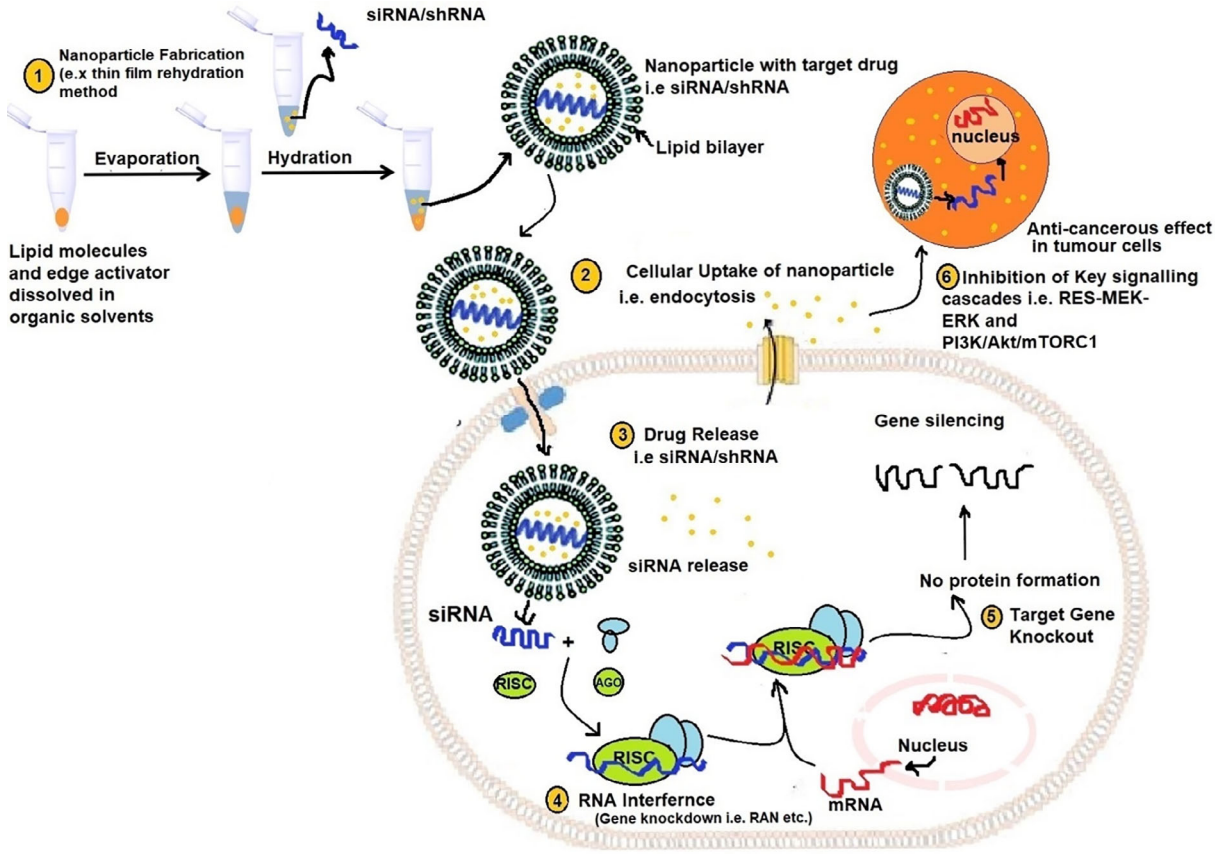

FIGURE 6 Anticancerous effect using siRNA/shRNA loaded nanoparticles via post-transcriptional gene silencing process (RNAi)

dicer and TRBP/PACT complex, which causes the loop of the shRNA get processed off and double-stranded siRNA with 2 nt 3′ overhang is formed. This double-stranded siRNA is then used in posttranscriptional gene silencing process as discussed above in siRNA delivery in this review. This siRNA will either induce RNAi by destroying target mRNA or through translational suppression via processing bodies (or p-bodies).

It appears from the recent studies that shRNA is superior to siRNA in terms of more effective knocking down gene expression.[104] It has been shown in recent studies that shRNA has been delivered within the cell with the help of nanovehicles effectively to knockdown the expression of target mRNA. Unlike siRNA, where continuous administration of siRNA is required for effective knockdown of target gene, shRNA can be continuously synthesized by the host cell and hence continuous administration is not required.[18,102,104-109] This shows that shRNA is more durable and even a small dose of shRNA could provide improved efficacy. The ability of shRNA to knock down the gene responsible for metastasis or cancer increased its use against cancer/metastasis, which can well be observed from the recent studies.[102,105,110]

4.1 | shRNA delivery to cancer cells

shRNA delivery to tumor cells relies on a drug delivery system that not only protects it from the physiochemical environment of the body but also deliver it to the specific target site.[6] Although viral mediated delivery of shRNA against tumor has been done but toxicity remained the major problem.[111] Therefore, nonviral gene delivering agents are preferred over viral gene delivery system. Different formulations of nonviral gene delivery systems have been used to deliver shRNA within the cancer cells, especially in case of lung cancer and breast cancer cells, to reduce cancer growth and metastasis. It was found that in some cases of breast cancer that nuclear factor kappa B signaling pathway is overexpressed which can trigger metastasis[112] and blockade of this pathway may reduce or prevent metastasis. To prevent its overexpression, p65-specific shRNA incorporated with bioreducible Tween 85-s-s Polyethyleneimine 2K (TSP) were used successfully.[112] The TSP NPs were proved very effective in inducing apoptosis in cancer cells along with the prevention of proliferation and invasion of targeted cells. TSP driven nanovehicle seems very effective delivery system because of its stable nature, which is imparted by the Tween molecule present in the NP formulation. This also suggests that these NP had long circulation time, which helped them to retain shRNA in the body environment for long time. In addition to this, Tween also assists in increasing cellular uptake by interacting with low-density lipoprotein receptors on the cell surface. Moreover, the disulfide bond present in the NPs was probably responsible for inducing rapid release of shRNA in an intracellular cancer environment of high concentration of glutathione.[112] It may be

assumed that TSP driven NP can be used to deliver shRNA successfully for cancer treatment. In another instance where shRNA encapsulated within the functionalized gold NP was delivered successfully to the target cancer cell and significant results were observed is also reported.[111] Apart from easily modifying their functional groups for target-specific delivery, bioinert, and nontoxic nature of gold NP were their major advantage and which greatly helped them to deliver shRNA more effectively. More than 85% of the target gene (p53) knockdown was observed.[83] In another occasion, folate-chitosan graft PEI was conjugated with shRNA and delivered to suppress lung tumor angiogenesis by blocking Akt signaling pathway.[113] Although this category of nanodelivery vehicle has shown significantly less toxicity and high transfection efficiency, its limited cell specificity remained a hurdle. Later, it was hypothesized that molecular size of PEI is directly proportional to the cell toxicity, that is, the bigger the molecular size of PEI, the greater the chances of toxicity. Unlike PEI, chitosan was proved less toxic and more biocompatible than PEI. But overall nature of nanovehicles, which were composed of chitosan and PEI were found less toxic and later reasoned that chitosan folate species may have shielded the charge on PEI, hence less toxicity. Therefore, PEI-based drug delivery vehicle can be improved if we could minimize their molecular weight and shield their charge with the support of other molecular species without compromising their previous delivering properties. It has been attempted to incorporate chemotherapeutics in order to improve the cancer treatment, for instance, Pluronic P85-PEI/D-α-tocopheryl polyethylene glycol 1000 succinate (TGPS complex) or PTPN NPs were used to carry shRNA along with an anticancerous chemical drug called paclitaxel for the treatment of lung cancer.[109,114] The reduction followed by inhibition of metastasis was observed and metastasis was inhibited to 88% whereas the growth of the cancer cell was inhibited by 91%.[62] Growing understanding of biochemical pathways may help us to fabricate improved delivery system for shRNA but may also improve the chances of treatment. The ongoing studies on shRNA delivery suggests that shRNA is a very useful tool to treat different forms of cancers and could become moreeffective if improved drug delivery system is used.

5 | CONCLUSIONS AND FUTURE PROSPECTS

The successful clinical trials in most studies show that RNAi mediated drugs are the new and effective method of cancer treatment. This method has shown progress and effective results in knock down of cancer causing genes. To summarize, it is hypothesized that RNAi-based therapy will be used in future for cancer therapy. Moving forward, nanoparticulate delivery systems derived from block co polymers, lipids, lipids conjugates, or others can be fabricated. Various novel drug delivery systems made-up via innovative methods have been used by researchers for intracellular siRNA delivery. The novel properties of siRNA/shRNA allowing them to knockout the expression of target mRNA through the RNA interference process (also known as post-transcriptional gene silencing process) and makes them extremely useful tool in cancer treatment, especially in lung and breast cancer. As siRNA/shRNA cannot be delivered naked, a better delivery system is therefore required which can deliver them without compromising its delivering properties. This will allow the effects of siRNA/shRNA on cancers cells as antimetastatic biotherapeutics can be studied in detail. In most of the cancer cells, especially in breast and lung cancer cells, the hyperactivation of the RAS/MEK/ERK and PI3k/AkT/mTOR pathways have been observed. This overexpression can be inhibited by using the Ran interference process with the help of siRNA/shRNA. Therefore, improved delivery system which could deliver siRNA/shRNA to knockdown the gene responsible for metastasis in breast cancer as well as lung cancer patients is required and the conceptual understanding of biological responses toward the nanoparticulate delivery system may help us to manufacture a better delivery system and could provide an improved and more efficient treatment against malignant tumors, especially in lung and breast cancer cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

AS and NKJ designed and wrote the manuscript. Rest of the authors coordinated and drafted the manuscript. All authors read and approved the final manuscript.

ETHICAL STATEMENT

Not Applicable

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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