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Advances in RNAi-based nanoformulations: revolutionizing crop protection and stress tolerance in agriculture

Shivangi Mathur,† Ambika Chaturvedi† and Rajiv Ranjan *

Nucleic acid-based therapeutics have the ability to tackle a wide range of diseases and stress tolerance that present significant obstacles for conventional approaches in agriculture. RNA-based medicines have become a promising approach, using nanoformulation treatments to specifically target certain diseases. Nanoformulations offer numerous benefits in comparison to alternative treatment methods, such as precise administration, minimal toxicity, and medication loading compatibility due to their bioactivity. There are a variety of nanoformulations available today, such as liposomes, polymeric nanoparticles (NPs), magnetic NPs, nanogels, and solid lipid nanoparticles (SLNs). RNA-based therapy employs intracellular gene nanoparticles containing messenger RNA (mRNA), which play an important role in stress management and pest as well as disease control. The adoption of mRNA-based technology paves the way for future technological progress. This review focuses on elucidating the process underlying the development of RNA interference (RNAi) and the diverse array of nanocarriers employed for the transportation of RNAi. Currently, this technique is being employed in the field of crop protection to combat diseases, pests, and environmental stress. The article highlights the benefits of RNAi mediated nanoformulations and discusses the significant obstacles that must be overcome to improve the viability of this technology for future applications.

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1. Introduction

The growing challenges posed by diseases, pests, and environmental stresses in agriculture necessitate innovative and sustainable approaches to crop protection and enhancement. Traditional methods such as chemical pesticides and genetic modifications, while effective, face limitations related to environmental impact, resistance development, and public acceptance. In this context, advances in biotechnology have opened new horizons for addressing these issues at the molecular level. One of the most cutting-edge biotechnological advances in medicine is gene therapy.[1] The modification of defective or disease-causing genes involves introducing genetic medication, such as DNA or RNA, into an individual's or plant's cells to deactivate, replace, or repair them is called gene therapy. In 1989, the first successful attempt was made to manipulate

Plant Molecular Biology Lab, Department of Botany, Dayalbagh Educational Institute, Dayalbagh, Agra 282005, India. E-mail: rajivranjan@dei.ac.in

† Both the authors contributed equally.

Shivangi Mathur

Ms Shivangi Mathur is currently pursuing a Ph.D. under the supervision of Dr Rajiv Ranjan at the Department of Botany, Dayalbagh Educational Institute, Agra. Her research focuses on the effect of various elicitors on secondary metabolite production in medicinal plants and exploring sustainable approaches for enhancing bioactive compounds in plant-based medicine.

Ambika Chaturvedi

Ms Ambika Chaturvedi is pursuing a Ph.D. under the supervision of Dr Rajiv Ranjan at the Department of Botany of Dayalbagh Educational Institute, Agra, India. She is a researcher specializing in molecular plant pathology. Her work focuses on RNA interference (RNAi)-based plant disease management and nanotechnology applications for plant protection.

human genetic information by directly inserting DNA into the nuclear genome, which marked the introduction of gene manipulation.[2,3] Subsequently, several investigations developed diverse kinds of gene-based medications.[4] Nucleic acid-based medicines can be generically classified as RNA, DNA, and artificial nucleotide (XNA) based pharmaceuticals. DNA aptamers, antisense oligonucleotides, DNA and plasmids are some of the examples of DNA-based medications. Similarly, RNA-based pharmaceuticals can be further classified as aptamer-based medications, mRNA, shRNA, RNA-based antisense oligonucleotides, RNAi (siRNA and miRNA), ribozymes, and mRNA. XNA-based gene therapies primarily belong to the class of antisense oligonucleotide-based medicines.[5–7]

Gene therapies also include the CRISPR/Cas9 system, stem cell therapy, and CAR T-cell therapy (chimeric antigen receptor T) for cancer.[8] The three primary ways that gene therapy functions are: (i) replacing a disease-causing gene with a healthy copy; (ii) inactivation of disease associated genes; and (iii) the insertion of a modified gene into the body to treat a disease.[9] Research is being done on the use of nucleic acid-based medicines to cure a variety of illnesses, including various cancers, cardiac conditions, viral infections, and hereditary illnesses that are not common.[10–12] As of 2022, approximately 39 gene medicines had been licensed, and numerous others were undergoing varying stages of clinical studies.[13] The simplicity of siRNA characterization and manufacture, however, led to a greater focus on siRNA-based gene therapy. The small size of siRNAs facilitates changes that increase their nuclease stability. Additionally, siRNAs can be synthesized in vast quantities.[14]

The advent of catalytic RNA introduced a novel approach to addressing a wide array of significant human ailments, such as malignancies and Alzheimer's disease. The emergence of RNA interference (RNAi) approaches utilizing double-stranded RNA (dsRNA) to suppress genes in nematodes was observed in the late twentieth century.[15,16] Natural gene expression regulation *via* RNA interference (RNAi) has become a potent method for gene expression manipulation in several fields, such as transgenic design, functional genomics, and drug assessment.[16,17] siRNAs are being more commonly employed as sequence-specific inhibitors of transcription. They are used to specifically target cells and induce gene silencing in the post-transcriptional phase of protein production by selectively deleting messenger RNAs (mRNAs). It is feasible to specifically target any gene responsible for causing a disease, as well as any specific cell type or tissue. In addition, siRNA-based gene therapies are extensively researched for a range of diseases. Furthermore, the FDA has already approved five siRNA-based drugs to treat conditions such as acute hepatic porphyria (AHP), hereditary transthyretin amyloidosis (hATTR), primary hypercholesterolemia, primary hyperoxaluria type 1 (PH1), and amyloid transthyretin-mediated (ATTR) amyloidosis.[18–22]

The majority of RNAi research has historically focused on human diseases, but recent findings indicate that it may also be useful to study plant diseases and improve crop yields. RNAi technology has been used for gene functional studies as well as for double-stranded RNA (dsRNA) applications and has shown great promise in modifying plant processes and improving crop protection against viruses, fungi, and insects by targeting specific genes.[23–25] DICER-LIKE proteins catalyze the intracellular processing of double-stranded RNA (dsRNA) into siRNAs (small interfering RNAs). These are recognized by Argonaute (AGO) proteins, leading to the formation of RNA-induced silencing complexes (RISCs).[26] A RISC, in conjunction with small interfering RNAs (siRNAs), binds to mRNA molecules that have complementary sequences, which facilitates the cleavage of the target mRNA and inhibition of its translation.[15] Technologies based on RNA interference are thought to provide little risk and have the potential to decrease the use of chemical pesticides to achieve sustainability objectives. Several studies suggest that the surface application of dsRNA can be used to control plant viruses including bean common mosaic virus, plant viruses including pepper mottle virus, and tobacco mosaic virus (TMV).[27–30]

The biodegradability of RNA is a substantial obstacle for the implementation of RNAi in crops, as it provides a limited window of protection lasting only a few days.[31] In order to achieve long-term and efficient RNA interference, dsRNA molecules need to be carefully delivered into plant cells while being shielded from environmental deterioration. Current research has concentrated chiefly on nanoparticles as intelligent carriers of RNA interference (RNAi) molecules to achieve protection against diseases.[32,33] The physiological and intracellular barriers of bare siRNA preclude its use as a drug candidate in clinical applications. Multiple organizations collaborated to tackle the difficulties and successfully overcome some obstacles related to siRNA delivery. Safe delivery of siRNA for clinical applications has been achieved using a variety of techniques. This review paper focuses particularly on the use of RNAi in diseases, pests, and environmental stresses in agriculture and provides current insights and suggests future prospects for investigation. This paper addresses the molecular aspects and

Rajiv Ranjan

Dr Rajiv Ranjan is currently working as an Associate Professor at the Department of Botany, Dayalbagh Educational Institute, Dayalbagh, Agra, India. He obtained his Ph.D. from the Institute of Life Sciences, Bhubaneshar, India. He is an active researcher in the field of plant virology, synthetic biology in addition to transcriptomics of medicinal plants. He is a recipient of several fellowships from ICAR, DBT, CSIR, and INSA during his academic career. He has to his credit published more than 80 international scientific papers including book chapters. He has completed five research and development projects from national funding agencies. Dr Ranjan also visited the USA, Canada and Switzerland as a part of conferences and research projects. Five Ph.D. degrees have been awarded under his guidance and he is currently guiding six Doctoral Research Scholars.

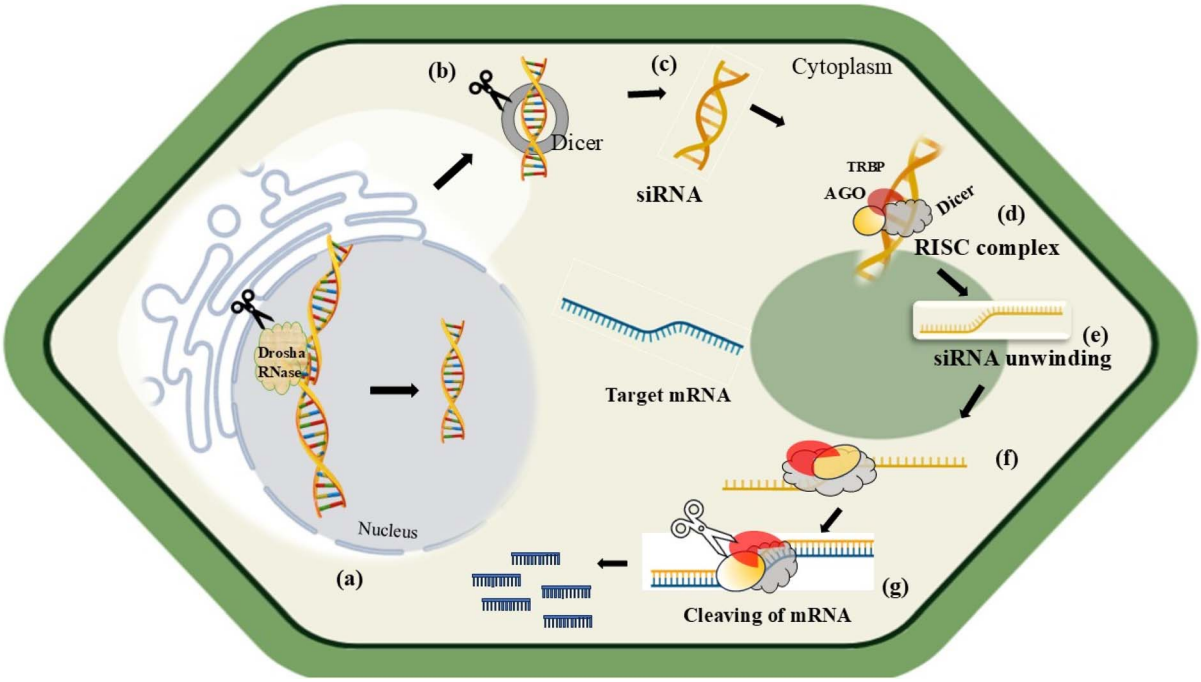

Fig. 1 A schematic representation of the RNAi processing of (a) long double-stranded DNA by Drosha RNase, enzyme Drosha, and export of short dsRNA to the cytoplasm, (b) binding of Dicer leads to the fragmentation of dsRNA into (c) small interfering RNAs (siRNA) of 21–25 bp in length, (d) recruitment of siRNA by the RISC-protein complex, and (e) unwinding of siRNA, and (f) binding of siRNA to target mRNA, which facilitates (g) cleaving of mRNA and leads to the formation of disrupted protein.

difficulties related to the delivery of siRNA and potentially explores the possible solution of designing and developing siRNA delivery systems.

2. Elucidation of the RNAi cascade

RNA interference is classified as an endogenous cellular mechanism that works to modulate the expression of the target gene by suppressing its translation. RNAi works as a guard intracellularly from invading nucleic acids like viruses and transposons. RNA silencing is necessary for defence against diseases in eukaryotes and has been applied in biotechnology to create resistance to various diseases. The key players in gene regulation are small interfering RNA (siRNA), microRNA (miRNA), and short hairpin RNA (shRNA). siRNA is introduced exogenously or can also be generated from long double-stranded RNA (dsRNA) *via* the action of an enzyme Dicer, to downregulate the gene expression, whether is miRNA produced endogenously within the cell. The siRNA molecule is incorporated into the protein complex, namely RISC (RNA-Induced Silencing Complex) as given in Fig. 1. The target nucleic acid and the effector proteins are the core components of the RNAi

Table 1 The list of approved siRNA medications

Approved siRNA medications	Treatments	Mechanism	References
Patisiran	Inherited transthyretin-mediated amyloidosis polyneuropathy in adults	Production of TTR (transthyretin) protein	38
Givosiran	Acute hepatic porphyria in adults	Reduce the production of toxic precursors of heme, such as PBG (porphobilinogen) and ALA (aminolevulinic acid)	39
Inclisiran	Primary hypercholesterolemia or mixed dyslipidemia	Inhibition of the production of proprotein convertase subtilisin facilitated LDL-cholesterol level reduction	40
Lumasiran	Primary hyperoxaluria type 1 in adults and pediatric patients	Decrease the oxalate hepatic production through the inhibition of glycolate oxidase	41

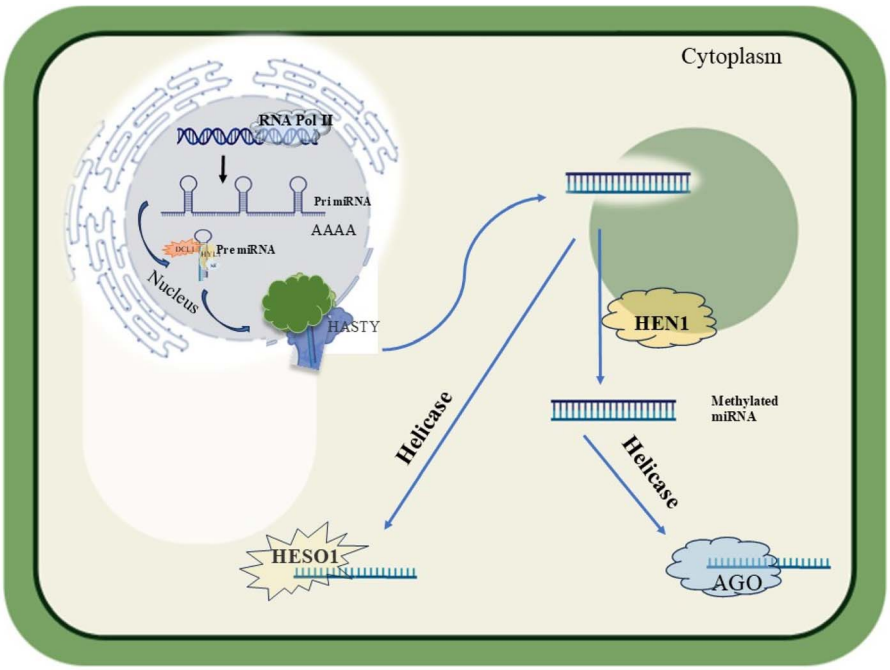

Fig. 2 Illustration of the miRNA biogenesis pathway for producing mature miRNA. Pol II transcribes MIR genes in plants, which are contained in the noncoding regions between protein-coding genes. With the help of HYL1/DRB1 and SERRATE (SE), the nuclear RNase III Dicer-like1 (DCL1) produces plant miRNAs. DCL1 carries out both stages of miRNA processing to create the miRNA/miRNA duplex, which is transported by HASTY to the cytoplasm. HEN1 modifies the miRNA/miRNA duplex at the 3′ end *via* 2′-*O*-methylation. The second mature miRNA gets loaded onto RISC and includes the AGO protein. The poly(U) polymerase HESO1 uridylates the 3′ terminal of unmethylated plant miRNAs, which leads to their eventual destruction.

system. The effector protein of the system facilitates target-specific recognition for silencing. The RISC protein is composed of a group of effector proteins that are part of the Argonaute protein.[34] The complex consists of two strands; one is a guided strand and the other is a passenger strand. In small RNA, the guide strand recognizes its target mRNA, and this recognition is facilitated by partial complementarity that occurs between the guide strand and the mRNA, and simultaneously the passenger strand is degraded. The perfect complementarity between the guide strand and target mRNA leads to the introduction of cleavage within the RISC factor. RNA duplexes bigger than 21 base pairs interact with the enzyme Dicer, a type of RNase III endonuclease family enzyme, facilitating their cleavage and subsequent transfer to the RISC loading complex (RLC) and further activating the RNA interference (RNAi) pathway. Small interfering RNAs that are fewer than 21 base pairs in length can avoid Dicer cleavage and directly reach the RISC through interactions. TAR RNA-binding protein (TRBP) facilitates these interactions, with Dicer playing a role in both pathways. However, the second pathway can also function independently of Dicer.[35,36]

RNAi-based therapeutics are used in eukaryotes and represent a significant development in biotechnology and medicine. As the research continues, RNAi is revealed as a key player for the future of medicine and innovative disease management strategies in agricultural practices. Ongoing research in a particular field continues to uncover various ingenious methods for RNAi delivery that provide efficacious outcomes. The thriving new era of RNAi therapeutics began with the approval of Patisiran, in which promising RNAi drugs and clinical delivery strategies are moving toward more advanced clinical research trials and development.[37] RNAi can be synthesized by various methods including *in vitro* methods *via* using purified enzymes and *in vivo* methods through genetically modified bacteria. Both methods utilize the involvement of DNA-dependent RNA polymerase from bacteriophage T7, which facilitates the transcription of the target sequence present downstream of the promoter.

With the approval of four siRNA medications by the Food and Drug Administration, the research is continuing toward the advancement of RNAi-based therapeutics for enhanced efficacy and stability. With the increasing practices toward enhancing the efficacy and stability of RNAi, the risk of the safety issue generated due to targeting exogenous siRNA also increases (Table 1).

miRNA is an endogenous post-translation regulator, which could be mRNA degradation or translation repression that is derived from the long precursor, *i.e.* pre-miRNA. The long precursor molecule then transfers into the cytoplasm, where Dicer, an RNase III, cleaves the precursor to generate 22–23 nucleotide long mature miRNA (Fig. 2). For downregulation of target gene expression, it binds to the 3′ UTR region.[42,43] miRNA can also be designed for disease resistance and delivered using an appropriate carrier. Various tools are also available to customize the miRNA with off-targeting filters,[44] such as miRbase,[45] RNA22,[46] OptiMiR,[47] miRNAFold,[48] *etc.*

3. Strategies of RNAi delivery using nanocarriers

Nanotechnology is a miniaturized, cost-effective, and multidisciplinary scientific field whose aim is to enhance the quality of society. Nanotechnology involves the incorporation of particles of a nanometer in size. Nanotechnology broadly focuses on atomic and molecular levels of affecting matter. Nanotechnology research can be classified into numerous branches such as nanobiotechnology, nanomedicines, nanobiopharmaceuticals, nanobiomechanical, *etc.* Nanotechnology can be utilized in diverse branches including textiles, biotechnology, cosmetics, agriculture, electronics, energy storage, security, *etc.*[49] Nanotechnology is also proven to improve agriculture quality by providing resistance to various pests and diseases such as ZnO NPs and bioactive protein-loaded carboxymethyl chitosan NPs, which can be used against plant bacterial diseases, humic acid-coated Fe_3O_4 nanoparticles and ZnO NPs against fungi.[50,51] Over the last few decades, the research on the potential of siRNA therapeutics increased but certain limiting factors remain such as toxicity, short half-life, off-targeting, and degradation in lysosomes and to overcome these issues there is a requirement for an ideal siRNA delivery system.[52] Based on extensive research, nanoparticles can be considered a promising tool for dsRNA delivery. The miniature size of nanoparticles enables the encapsulation of small RNA molecules and also safeguards them from degradation.

3.1. Lipid nanoparticles

Extensive studies prove that lipid nanoparticles (LNPs) are efficient tools for delivering small molecules, drugs, siRNA, and mRNA. LNPs were also used as a delivery vehicle for antigen mRNA (mRNA-1273 and BNT162b) against coronavirus.[53–56] Lipids used as delivery vehicles are categorized as cationic or ionizable lipids. The positive charge characterizes cationic lipids. Cationic nanoparticles possess a specific ability to induce the permeability of plasma membranes through the production of nanoscale holes, so they can be considered a promising tool in nanotherapeutics. For instance, Hsu and colleagues used a cationic lipid nanoparticle formulation as a delivery agent for miR-122 delivery for restoration of gene expression in hepatocellular carcinoma.[57] mRNA-encapsulated cationic lipid nanoparticles are an efficient tool for the maturation of dendritic cells and T-cell proliferation against specific antigens. Lipid nanoparticles are a promising vehicle for delivering mRNA vaccines and facilitating lymphoma growth reduction.[58] Traditionally, cationic lipid nanoparticles are characterized by the positive charge head, and on the other hand, ionizable lipid nanoparticles' charge properties depend upon the pH. When they are placed inside lysosomes or endosomes, they change their pH from neutral to acidic. This charge conversion property makes them an effective delivery agent for nucleic acids in intracellular compartments.[59–61]

3.2. Polymeric nanoparticles

Polymeric nanoparticles are nanosized particles and are composed of natural or synthetic polymers. Their special qualities and adaptability increase attention toward their use in various sectors such as drug delivery, medicine, environmental science, *etc.* They are primarily made up of biodegradable polymers such as poly(-lactic-*co*-glycolic acid) (PLGA), chitosan, polyethylene glycol (PEG), and others.[62,63] The conventional methods of nanoparticle synthesis are quite harmful. Nanoparticles also have various adverse effects and can accumulate in the system.[64] To cope with this, biodegradable NPs can be a promising tool[65] for agrochemicals and genetic material delivery because of their non-toxic and biocompatible nature. Biodegradable nanoparticles also show satisfactory adsorption ability.[66] Polymeric nanoparticles can be prepared by various methods depending on the physiochemical characteristics of drugs and application strategy. The preparation methods of these nanoparticles are divided into two distinct groups; one is based on the polymerization of monomers, whereas the other is primarily based on the use of preformed polymers. These methods can be further divided on the basis of the requirement of an emulsification system or not.[67,68] Polymeric nanoparticles have a significant ability to encapsulate drugs and provide target-specific delivery without harming drug stability. Polymeric nanoparticles are known for enhancing therapeutic efficiency while minimizing off-target effects.[69] The conjugation of polymeric nanoparticles with some specific agents also enhances the target delivery and encapsulation efficiency of siRNA and reduces toxicity. For instance, chitosan-coated Au nanoparticles, synthesized using layer-by-layer assembly through direct reduction, can be utilized to enhance the target delivery of siRNA. The presence of a gold core ensures colloidal stability, safeguarding siRNA, particularly when loaded onto chitosan. This approach is used to control *Helicoverpa armigera* by targeting the lipase and chitinase genes.[70,71]

When the siRNA is delivered *via* PLGA core nanoparticles coated with chitosan, it protects the siRNA from nuclease degradation and facilitates the fast release of siRNA into the cytosolic portion.[72] However, the cationic lipid coated PLGA nanoparticles can be considered as an effective formulation strategy to achieve sustained silencing of genes *in vivo*.[73] The nucleases present in blood can degrade siRNAs, which were further eliminated by the kidneys. Therefore, there is a requirement for an appropriate delivery set-up for the effectiveness of siRNA translation. Polyethylene glycol (PEG) can stabilize siRNA and protect it from degradation.[74] siRNA encapsulated in the copolymer of poly(ethylene glycol)-*b*-poly(D,L-lactide) (mPEG-PLA) with a cationic lipid can successfully escape from the endosome by facilitating remarkable gene downregulation. siRNA encapsulated in PEG-PLA successfully knocks down the luciferase gene and reduces tumor growth in the liver. The stability of siRNA-loaded PEG-monoacyl fatty acid can be enhanced when copolymerized with lipids.[75]

3.3. Inorganic nanoparticles

Inorganic nanoparticles are noncarbon-based versatile nanoparticles that include metal nanoparticles (silver, gold, and iron

oxide), quantum dots, carbon nanotubes, ceramic nanoparticles (titanium dioxide and zinc oxide), and silica nanoparticles. Inorganic nanoparticles are used extensively in biomedicines, electronics, and environmental sciences. However, proper handling of inorganic nanoparticles is necessary for the safety of the environment and human health.[76] They show appropriate surface chemistry and controlled size, allowing efficient siRNA encapsulation. Among explored inorganic nanoparticles, calcium phosphate (CaP) nanoparticles can form complexes that are stable with siRNA and show high biocompatibility. They are biodegradable and the ubiquitous presence of calcium phosphate in bones and teeth makes them non-immunogenic and can be explored as efficient choices for siRNA delivery.[77] However, the addition of arginine to CaP nanoparticles significantly increases siRNA binding without affecting particle size.[78] Furthermore, silica nanoparticles are known for their porous structure. The cationic silica coated-CaP nanoparticles are also proven as an efficient vehicle for the delivery of siRNA to suppress inflammation in monocytes, endothelial cells, and B-cells; however, their uptake is low in T-cells.[79] Similarly, gold nanoparticles provide high stability and reduce off-targeting effects. Moreover, small modifications in gold nanoparticles enhance cellular uptake and the gene silencing effect in cancer therapeutics.[80]

Quantum dots are another type of inorganic nanoparticle and enable ds-RNA delivery in plants and also increase the production of local and systemic siRNA.[81] The cell penetration ability and tunable properties of siRNA-loaded quantum dots significantly reduce the target gene transcription.[82] Due to their exceptional and distinctive properties—such as enhanced electron transfer, high aqueous solubility, increased photoluminescent quantum yield, minimal toxicity, resistance to photobleaching and photodecomposition, improved electrocatalytic activity, tunable fluorescence, excellent biocompatibility, long-term chemical stability, cost-effectiveness, and a high surface area-to-volume ratio—these materials have garnered significant interest in nanotechnology and biomedical science.[83] The β-secretase (BACE1) targeted siRNA loaded on fluorescent quantum dots (QDs) was reported to be significantly effective against β-amyloid (Aβ) in nerve cells. The positive charge of the QD-PEG/s complex facilitates the electrostatic attraction and efficient encapsulation of negatively charged siRNA. The coating of PEG protects the QDs from explosion in the intracellular environment and inhibits the toxic Cd^{2+} release; therefore, the QD-PEG/-siRNA nanocomplex is a more effective and less immunogenic carrier. Cationic polymers such as poly-L-lysine (PLL) are also known for the reduction of the toxicity of quantum dots.[84,85]

3.4 Carbon nanotubes (CNTs)

CNTs can be divided into the following categories: single-walled CNTs and multi-walled CNTs. CNTs with single walls have enormous potential both for protecting DNA from degradation in mammalian cells and in delivering DNA from RNAi or plasmids to plants. The cell wall of plant cells works as a limiting factor for CNT-mediated delivery. However, cup-stacked CNTs with cellulase can overcome the cell wall issue.[86–89] They are considered a potent vehicle for dsRNA delivery due to their tunable surface chemistry, exceptional tensile strength, and high aspect ratio. For instance, studies suggested that siRNA delivery using CNTs leads to successful gene knockdown of a target gene in both plants and animals. Additionally, CNTs show potential delivery in specific tissues such as the brain, cardiac muscles, *etc.*, by overcoming the cellular barrier. However, some cytotoxicity barriers and immunogenic issues are also related to CNTs mediating dsRNA delivery, which cannot be ignored, such as reactive oxygen species generation, photosynthesis inhibition, free radical accumulation, *etc.*[90,91]

3.5 Natural source derived nanocarriers

With the continued concern of life-threatening issues, the research towards natural source derived nanocarriers has expanded. Nanocarriers derived from natural sources offer high biocompatibility, low toxicity, and specific activity. Nanocarriers derived from natural sources include liposomes (derived from soybean lecithin), nanocrystals (from plant extracts), chitosan (from crustacean shells), alginate nanoparticles (from brown seaweed), albumin nanoparticles (blood plasma), lignin (from plant cell walls) and nanoemulsion (from natural emulsifying agents).[92–94] For instance, Foroutan and colleagues synthesized cerium oxide nanoparticles from *Caccinia macranthera* leaf extract with a size range of 30 nm and tested them for drug loading efficacy against cancer.[95] Bovine serum albumin is widely used for drug delivery due to its biocompatibility and is considered a promising tool for siRNA delivery.

3.6 Virus-like nanoparticles (VLPs)

The structure of VLPs is similar to that of viruses with an average diameter range from 2–200 nm but they lack a viral genome. Their high immunogenic nature facilitates its use in biomedicine and vaccine development.[51] VLPs are not only explored for vaccination but also proven effective for siRNA delivery like small interfering RNA (siRNA) loaded on brome mosaic virus (BMV) for breast tumor inhibition. The plant viral capsid plays a potential role in the delivery system, and the presence of specific ligands on the capsid surface increases the recognition of target tissues.[96] The genetic insertion and chemical modification can facilitate the modification of VLPs of the bacteriophage capsid, which enhances targeting. They also possess the capacity for rapid self-assembly in the presence of siRNA.[97] The conjugation of cell-penetrating peptides and chemically synthesized linkers with VLPs enables more efficient gene knockdown and endosomal escape, respectively.[98,99] However, the less stability under physiological conditions is the major drawback of VLPs, which can be overcome by cross-linking strategies for effective siRNA delivery.

3.7 Nanogels

Nanogels are crosslinked polymeric-based hydrogel nanoparticles that can be explored as next-generation drug delivery vehicles. They provide suitable features in the biomedicine field such as high drug encapsulation, uniformity, ease of preparation, low toxicity, and stability in serum. Their high-water content provides better encapsulation of biological molecules.

Their polymeric-based crosslinked structure provides controlled release of drugs.[100] Nanogels offer versatile delivery vehicles for RNAi delivery such as polyethylene-based core–shell nanogels, which are formed by a two-stage reaction and lead to the formation of highly uniform 40–200 nm nanosized particles. These nanogels are also effective in siRNA protection from enzymatic degradation. Crosslinked nanogels do not require any transfection agents for effective gene silencing.[101,102] The addition of pulmonary surfactant (PS) to nano-gels significantly increases their stability and enhances siRNA delivery in lungs both *in vitro* and *in vivo*. Research suggests that the surfactant protein B (SP-B) is a siRNA delivery enhancer in the proteolipid-coated hydrogel. Nanogels that are coated with polydopamine (PDA) provide siRNA-mediated low-temperature photothermal therapy, which provides damage-free therapeutic temperature through potential downregulation of Heat shock protein (Hsp). The direct intercalation between siRNA and nucleic acid bis-intercalator-based nanogel offers significant inhibition of tumor growth.[103,104]

4. Applications of RNAi mediated nanoformulations

4.1. Pest control

The global population is experiencing significant growth, necessitating a corresponding increase in food production to fulfil the rising demand. Approximately 18% of overall crop production is believed to be lost due to insect pests, with wheat and cotton being the most severely affected, experiencing respective losses of up to 50% and 80%. The global agricultural loss caused by insect pests is projected to be worth around US$470 billion, resulting in a significant financial penalty.[105] Furthermore, the efficacy of artificial chemical pesticides that have been introduced since the 1940s is consistently being compromised by the emergence of insect populations that are resistant to them. In addition, the lack of precision in synthetic pesticides is a significant problem. It is important to adequately consider the emergence of resistance in pest insect species to RNAi-based formulations when constructing delivery vectors. Imposing a significant selection pressure may result in mutations that alter the fundamental RNA interference apparatus.[106,107]

The purpose of RNAi-based biopesticides is to interfere with the expression of the target genes of agricultural diseases, such as fungi and other pathogenic microbes, insects, and weeds, in order to prevent their growth and lessen the harm they cause to crops. Consequently, new biopesticides with high specificity, simplicity, and efficiency can be developed *via* RNAi technology, which is predicted to spark yet another scientific and technological revolution and promote the growth of sustainable agriculture. Research organizations and agrochemical businesses have increased their research investments in order to translate the enormous potential of RNAi-based biopesticides into novel products.[108] Major RNAi patents have already been obtained by a number of prominent agrochemical and biotechnology businesses, including Syngenta, Corteva, and Bayer (Monsanto), which are utilising this technology to develop new products. Biopesticides based on RNA interference can be generally divided into two groups: (1) plant-incorporated protectants (PIPs) that are resistant to pests and diseases and (2) protectants (non-PIPs) that are not derived from plants.

In contrast, PIP RNAi-based biopesticides are innovative biopesticides that combine the benefits of resistant transgenic crops with RNAi technology. Currently, there are multiple products (VT4PRO in 2022, DP23211 in 2021, and MON87411 in 2017) that have received approval for commercialization. These products utilize the expression of dsRNA in genetically modified plants to manage pests. The U.S. Environmental Protection Agency (EPA) approved Monsanto/Bayer's genetically modified maize MON87411 in 2015. This maize is the first genetically modified crop to use RNAi technology. MON87411 was genetically modified to synthesize double-stranded RNAs targeting DvSnf7, the Cry3Bb1 protein with CRW activity, and the CP4 EPSPS protein linked with herbicide tolerance. This modification led to resistance against Coleopteran pests and tolerance to glyphosate. The EPA later granted approval for several hybrid products derived from MON87411, including SmartStax Pro (DAS-59122-7 × MON87411 × MON89034 × TC1507), Vorceed™ Enlist® (DP4114 × MON87411 × MON89034 × DAS-40278-9), VT4PRO™ (MON89034 × MIR162 × MON87411), and DP4114 × MON87411 Maize. Food from DP23211 has been approved by Food Standards Australia New Zealand (FSANZ).[109]

The genetically modified (GM) line was given by Dow AgroSciences (DAS). DP23211 maize has been modified genetically to produce dsRNAs targeting DvSSJ1, the herbicide-tolerant phosphinothricin acetyltransferase (PAT), an insecticidal protein known as IPD072Aa, and the selection marker phosphomannose isomerase (PMI) protein. This genetic modification confers resistance to *D. v. virgifera* and tolerance to the herbicide glufosinate-ammonium. In 2021, GreenLight filed for EPA approval of Calantha (foliar-applied dsRNA spray) to safeguard against the Colorado potato beetle. Ledprona, the active component of calendha, is a 490-bp dsRNA that targets the PSMB5 mRNA of *L. decemlineata*. In order to control varroa mites with dsRNA, Bayer filed for EPA registration of a spray RNAi biopesticide created with BioDirect technology in 2019. Additional BioDirect products are currently in the first phases of research, including a spray that specifically targets canola flea beetles with high effectiveness. Researchers are now developing RNAi based insecticides to target the diamond moth, Colorado potato beetle and varroa.[110] However, there is an urgent need for new RNAi products that may effectively combat a wider spectrum of insect species in the future. *Diabrotica virgifera* was among the initial and highly effective insects to be managed by the NT-mediated PIP approach. *D. v. virgifera* is an extremely destructive agricultural pest that results in economic losses of up to $1 billion each year in the United States. Remarkably, this insect has developed resistance to chemical pesticides, genetically modified Bt crops, and crop rotation tactics.[111] Nevertheless, this pest exhibits a high susceptibility to RNAi-based methods.

An RNAi system based on a star polycation (SPc) that is easily synthesized was found to be effective in suppressing gene

Table 2 Impact of various nanoparticle/dsRNA complexes on distinct insect species

Plant	Nanoparticles	Mode of delivery/time of exposure	Target	Results	References
Nicotiana benthamiana and *Solanum lycopersicum*	Carbon dots (CDs)	Low-pressure spraying method on plants with a concentration of 12 ng μL^{-1}	GFP transgenes	79% changes in phenotypic tissues by day 5 and a persistent bleaching phenotype for up to 20 days	115
N. benthamiana	Carbon nanotubes (CNTs)	Needleless syringe infiltration	mGFP5 transgenes	After infiltration, approximately 30% degradation of dsRNA-SNWT within 6 hours and 95% gene silencing in 1 day	116
Raphanus sativus	Star polycation (SPc)	Used a pneumatic water sprayer to spray (0.2 μL dsRNA/SPc)	ATP-A: LOC111039523; ATP-G: LOC111040044; ATP-d: LOC111041166	Degradation of dsRNA-SPc within 1.5 hours with about 50% control efficiency	117
A. thaliana	Cell-penetrating peptides (CPPs)	Needleless syringe infiltration (100 μL of the dsRNA-peptide)	GFP and firefly luciferase genes	Degradation of dsRNAs after 12 h while no silencing effects were observed; down-regulation was observed for dsRNA-peptides up to 2 days	118
S. lycopersicum	Layered double hydroxide (LDH)	Pollen drenching of LDH-50 and dsRNA (100 and 10 mg L^{-1})	Silences the target gene of CMV	Degradation of naked dsRNAs after 10 min and dsRNA-LDHs remain intact. 16.7% decrease in GUS protein activity.	119
N. benthamiana	Gold nanoparticles	Needleless syringe infiltration on mGFP5	Silences mGFP5 transgenes	After 30 min complete degradation of naked dsRNAs and dsRNA-gold NPs remain intact with low stress on plant tissue	120
—	Complex of carbon quantum dots (CQDs), chitosan, and lipofectamine2000	Efficient feeding delivery of all three nanoparticles by improving both dsRNA-CQD complex and cellular uptake in SSB larvae	Silence glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH) in rice striped stem borer (SSB)	Reduction in gene expression levels to about 40% in the gut, 44% in the other tissues, and the overall reduction in expression of the whole organism is 43%	121
N. benthamiana	Mesoporous silica nanoparticles (MSNs)	Infiltration	Magnesium chelatase genes and phytoene desaturase	Plant showed about 95% silencing efficiency	122
Arabidopsis thaliana	AuNPs	AuNP infiltration was performed by pressure infiltration	NPR1	Silencing of the gene within three days	123
Cotton	Mesoporous silica	Electrostatic interactions for efficient delivery into Sf9 cells	CYP6CY13 double-stranded RNA (dsRNA)	Reduction in *A. gossypii* improved by 19.95% in 5 days	124
N. benthamiana	Mesoporous silica nanoparticles	Agrobacterium-facilitated delivery	AC2 (dsAC2) gene	Threefold reduction of tomato leaf curl New Delhi virus	125
Rice seedling	Star polycations (SPc)	Spray induced delivery	hsc70-3 and PP-α	More than 60% mortality of white-backed planthopper *Sogatella furcifera*	126
—	Cationic chitosan nanoparticles	Leaf feed	*Helicoverpa armigera lipase* (JF999953) and *H. armigera chitinase* (AY326455)	About 2 fold downregulation in the target gene and about 5 fold suppression of the respective enzyme activity	127
Soybean seedlings	Spray method		ATPD, ATPE, TREH, and CHS1	Knockdown effects ranging from 86.86 to 58.87% and mortality up to 81.67%	128

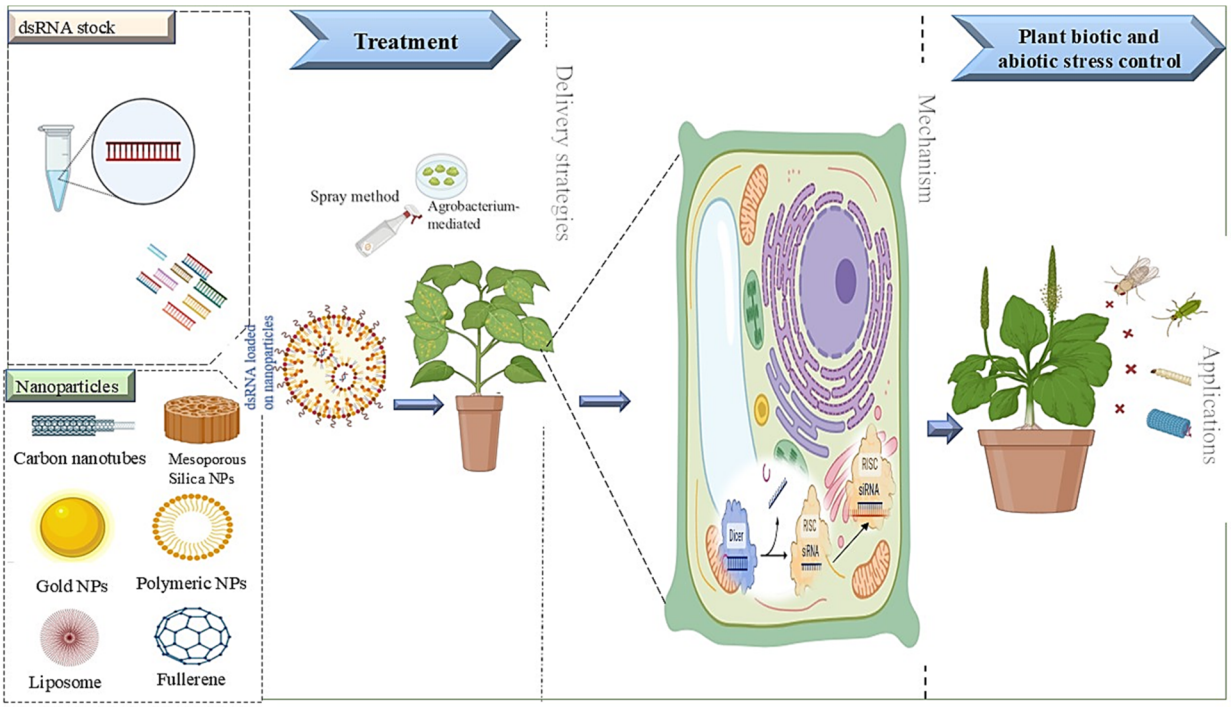

Fig. 3 A schematic representation of the processing of RNAi loaded on various nanoparticles and its mechanism of transfer in plants for use in agriculture.

expression in the RNAi-insensitive *S. frugiperda*. This can be applied topically, administered orally, or injected. When the eggs were placed in dsATP-d (100 ng μL^{-1}) or when dsATP-d alone (0.5 μg) was applied to the notum of 2nd instar larvae, no decrease in SfATP-d expression was observed. However, the expression of SfATP-d was reduced by more than 50% when treated with dsATP-d delivered by a readily synthesised star polycation (SPc). By providing oral feeding of 0.1 μg and 3 μg SPc-loaded dsATP-d, respectively, the expression of SfATP-d in the 4th and 6th instar larvae could be dramatically reduced. It was possible to effectively suppress the expression of SfATP-d in pupae and adults by injecting 10 μg SPc-loaded dsATP-d.[112] *Helicoverpa armigera* exhibits resistance to chemical pesticides and genetically modified organisms (GMOs). The non-transformative RNA interference method, which involves silencing certain genes by breaking down their mRNA using exogenous double-stranded (dsRNA) transport to *Helicoverpa*, encounters challenges related to degradation by nucleases and the acidic pH of the insect gut.[113] Chitosan nanoparticles (CNPs) efficiently facilitate the targeted transport of particular dsRNA molecules to suppress the expression of *Helicoverpa armigera* juvenile hormone methyltransferase (JHAMT) and acetylcholine esterase (ACHE) genes. Cationic CNPs, synthesized ionotropically, with a size of 100 nm and a charge of +32 mV, efficiently loaded dsRNA and effectively protected it against degradation by nucleases as well as the pH of the insect gut. The effective absorption of columnar insect gut cells was demonstrated by tagging CNPs with calcofluor fluorescence. The efficacy of using CNPs for delivering dsRNA was demonstrated by successfully suppressing green fluorescent protein in Sf9 cells.[114] The various types of RNAi based nanoformulations used for controlling pests are mentioned in Table 2.

4.2 Stress tolerance

The diverse biotic and abiotic stresses contribute to a decline in agricultural productivity. Biotic pressures mostly arise from nematodes, viruses, insects, bacteria, parasitic weeds, and fungal diseases. These factors have a significant impact on plant physiology and growth, resulting in a reduction in overall agricultural output.[129] Additionally, the management of pests is a significant obstacle in several agricultural systems. Plants possess innate defense mechanisms to combat infections produced by bacteria, viruses, and fungal pathogens. However, certain diseases provide significant challenges in terms of control and management.

Various abiotic stimuli, including drought, cold, freezing, heat, and salinity, hinder the morphological, physiological, and molecular responses, resulting in significant growth abnormalities. Certain genetic networks linked to tolerance are activated by plant diseases, which produce an environment similar to that of an abiotic stress, offering either full or partial protection. For instance, genes responsive to oxidative stress, wound healing, and dehydration are activated by root diseases.[130] According to Lamers *et al.* (2020) and dos Santos *et al.* (2022), plants under stress typically exhibit leaf chlorosis, destruction of photosynthetic machinery, decreased water potential of the leaves, decreased turgor pressure, nutrient deficiency, surged oxidative damage, and improper osmotic balance, which can ultimately result in dying cells or tissues,

stunted growth, wilting, premature or delayed maturity, and improper floral and fruit development.[131,132] Various types of stress activate distinct groups of genes, but there is some overlap among these genes. Ultimately, these genes produce a controlled response to mitigate stress-related challenges.[133] However, in daily life, it is highly unlikely that a plant will only encounter a single stressful event at any given time. Plants must therefore control the expression of multiple genes to create a complex network of biochemical pathways and effector proteins in order to respond to multiple stress events.[134]

Plants have a variety of defense systems to fend off biotic and abiotic stresses and survive extreme circumstances. Plants tend to accumulate a large amount of suitable osmolytes, such as glycine betaine, proline and soluble carbohydrates, in response to abiotic stress. Furthermore, these chemicals not only possess the capability to detect stress, but they also play a crucial role in maintaining osmotic balance, preserving the integrity of cell membranes, and safeguarding intracellular proteins and enzymes.[135] In addition, these chemicals play a significant role in the tolerance mechanism by detoxifying reactive oxygen species and protecting the cell membrane and photosynthetic machinery from damage.[136]

Recently, biotic stress, particularly from insect pests, has had a major impact on food crops. The escalating use of pesticides poses a significant threat to our ecosystem. Hence, nanoparticles have the potential to serve as a vehicle for enhancing the efficacy of industrial pesticides (Fig. 3). Ag@dsDNA@GO refers to silver nanoparticles that are directed by DNA and graphene oxide (GO). These nanoparticles effectively reduce the harmful impacts of *Xanthomonas perforans* on tomatoes.[137] Furthermore, Ag nanoparticles were discovered to possess deadly properties against soil-borne diseases, including nematodes. The *Serratia* sp. of plant growth-promoting rhizobacteria (PGRB) has the ability to produce Ag nanoparticles through biosynthesis. *Bipolaris sorokiniana*, a spot blotch pathogen that affects wheat plants, became less harmful when PGRB *Serratia* sp. was employed in a greenhouse setting.[138] The environmentally friendly production of silver nanoparticles was discovered to be successful in combating many types of fungi, including *Gloeophyllum abietinum*, *Chaetomium globosum*, *Gloeophyllum trabeum* and *Phanerochaete sordida*. Tomato plants were subjected to greenhouse conditions and treated with photoactive TiO_2 NPs that possess a high level of photocatalytic activity. The work focused on the significant antibacterial properties of TiO_2 NPs against *X. perforans*, a pathogen of bacterial spot disease.[139]

5. Advantages and challenges of RNAi mediated nanoformulations

The RNAi technology is a revolutionary method that has the potential to impact the agriculture sector greatly by providing unmatched capabilities for controlling pests and managing plant diseases. The evolution of RNAi has been strongly linked to the progress made in complementary technologies, such as the use of vectors for the recombinant generation of RNAi and the micro/nanoencapsulation of si/dsRNA.[140] At first, the high production costs and stability issues were the concerns. However, subsequent advancements have dealt successfully with these obstacles, making it possible for the technology to be widely adopted. An important factor that has advanced the discipline is the implementation of encapsulation techniques. These techniques, which involve using various substances such as liposomes, virus-like particles, polyplex nanoparticles, and bioclay, create a protective barrier that prevents the breakdown of RNAi molecules. This improves the stability of the molecules and makes it easier for cells to absorb them. Encapsulation preserves the integrity of the RNAi payload, thus prolonging its lifespan and improving its efficacy in fighting against certain pathogens or diseases.[141]

Furthermore, RNAi-based technologies offer numerous advantages compared to traditional methods. By utilising a non-transgenic method, issues about public acceptance and regulatory approval are reduced, which enhances trust in the technological implementation. The increased specificity of RNAi molecules, designed to selectively target certain pathogens, greatly decreases the likelihood of off-target effects, thus improving accuracy and minimizing unwanted outcomes.[142] Precision is especially vital in the fight against viral illnesses, as traditional treatments frequently prove inadequate. Moreover, the short-lived nature of dsRNA molecules helps address concerns about residues, effectively mitigating food safety and environmental issues associated with conventional chemical pesticides. Additionally, the reduced likelihood of resistance development due to the variability in DICER enzyme cleavage sites underscores the long-term sustainability of RNAi-based approaches.[143]

After being administered systemically, siRNA formulations face obstacles such as renal clearance, uptake by phagocytes, interaction with serum proteins, and destruction by nucleases in order to reach the intended target site. Despite attempts to overcome these challenges by modifying siRNA molecules, such as using naked siRNAs, nonviral vectors, or viral vectors, their effectiveness in laboratory settings often differs from their performance in living organisms due to the numerous impediments encountered before reaching the intended cells. The route of delivery is crucial, as topical therapy is appropriate for exterior tissues like the eye and skin, while intravenous injection is necessary for internal conditions that cannot be treated topically, such as tumors. Therefore, it is necessary to develop techniques to improve the stability and circulation period of siRNA in the bloodstream.

Effective transport of siRNA is hindered by intracellular barriers, including endosomal trapping, immunological stimulation, and off-target effects, which present substantial challenges. SiRNAs need to overcome physiological obstacles and escape endosomal entrapment in order to reach the cytoplasm and initiate RNAi mechanisms. In addition, immunological activation poses the risk of activating the innate immune system, which could result in severe effects if the dosage of siRNA is too high.[144] Moreover, the brief siRNA sequences enhance the probability of off-target effects, where undesired genes homologous with the target gene may be unintentionally silenced. This emphasizes the necessity for accurate targeting

tactics to minimize unintended consequences. Although there are limitations, progress in siRNA delivery shows potential for transforming gene therapy, as long as efficient methods are created to overcome the complex set of physiological and intracellular obstacles faced in living organisms.[145]

6. Conclusion and future perspectives

In conclusion, RNA interference (RNAi) technology, particularly when implemented through nanoformulations, holds immense promise in agriculture. Advancements in complementary technologies have addressed initial concerns about production costs and stability, facilitating widespread adoption. Encapsulation techniques have been instrumental in preserving RNAi molecule integrity, extending their efficacy in combating pathogens. RNAi techniques provide several benefits compared to conventional methods, such as increased precision and decreased environmental implications. However, challenges persist in systemic administration of siRNA formulations, requiring strategies to enhance stability and circulation. Despite the hurdles, advancements in siRNA delivery hold the potential to revolutionize gene therapy, provided barriers are overcome.

Pharmacokinetics (PK) and pharmacodynamics (PD) are new concepts of RNAi nanoformulations, which play a pivotal role in determining their efficacy in agricultural applications. PK parameters such as stability, distribution, and degradation within plant or pest systems directly impact the ability of RNA molecules to reach their intended targets in sufficient concentrations. For example, nanoformulation strategies like surface modifications with polyethylene glycol (PEG) or ligand-based targeting can enhance stability and delivery efficiency by protecting RNAi from enzymatic degradation. On the other hand, PD focuses on how RNAi molecules interact with biological targets to achieve effective gene silencing, including dose-response relationships and the duration of silencing effects. Sustained release mechanisms from nanocarriers have been shown to improve the persistence of RNAi activity, which is critical for prolonged pest control or stress resistance in crops. Integrating PK and PD insights into nanoformulation design can optimize RNAi technologies for improved efficiency, reduced environmental impact, and enhanced applicability in sustainable agriculture.

Ongoing concerns include production scalability and environmental safety, demanding collaborative efforts to reduce costs and assess risks. Through innovation and evaluation, RNAi technology offers transformative potential for sustainable pest and disease management. To fully realize siRNA-based gene therapies' potential, addressing remaining barriers through comprehensive research and strategic interventions is essential. Furthermore, multidisciplinary alternatives integrating RNAi-mediated gene silencing are anticipated, such as elicitation and metabolic control techniques in crop resistance development. These technologies, including elicitor nanoparticles, utilize dual control mechanisms to counteract pathogens. Overcoming manufacturing expenses remains a challenge, highlighting the role of biotechnology in enhancing profitability.

Abbreviations

NPs	Nanoparticles
SLNs	Solid lipid nanoparticles
RNAi	RNA interference
XNA	Artificial nucleotide
dsRNA	Double-stranded RNA
AGO	Argonaute proteins
siRNAs	Small interfering RNAs
LNPs	Lipid nanoparticles
CNPs	Chitosan nanoparticles
CNTs	Carbon nanotubes
DDSs	Drug delivery systems

Data availability

No primary research results, software or code have been included and no new data were generated or analyzed as part of this review

Author contributions

SM and AC wrote the manuscript and RR edited the manuscript.

Conflicts of interest

The authors report there are no competing interests to declare.

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