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Short interfering RNA (siRNA)-Based Therapeutics for Cartilage Diseases

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Abstract

Articular cartilage injury, as a hallmark of arthritic diseases, is difficult to repair and causes joint pain, stiffness, and loss of mobility. Over the years, the most significant problems for the drug-based treatment of arthritis have been related to drug administration and delivery. In recent years, much research has been devoted to developing new strategies for repairing or regenerating the damaged osteoarticular tissue. The RNA interference (RNAi) has been suggested to have the potential for implementation in targeted therapy in which the faulty gene can be edited by delivering its complementary Short Interfering RNA (siRNA) at the post-transcriptional stage. The successful editing of a specific gene by the delivered siRNA might slow or halt osteoarthritic diseases without side effects caused by chemical inhibitors. However, cartilage siRNA delivery remains a challenging objective because cartilage is an avascular and very dense tissue with very low permeability. Furthermore, RNA is prone to degradation by serum nucleases (such as RNase H and RNase A) due to an extra hydroxyl group in its phosphodiester backbone. Therefore, successful delivery is the first and most crucial requirement for efficient RNAi therapy. Nanomaterials have emerged as highly advantage tools for these studies, as they can be engineered to protect siRNA from degrading, address barriers in siRNA delivery to joints, and target specific cells. This review will discuss recent breakthroughs of different siRNA delivery technologies for cartilage diseases.

Lay Summary

Articular cartilage breakdown is a hallmark of osteoarthritis, which is difficult to repair and treat. Currently, there is no disease-modifying therapeutic approved by FDA to treat osteoarthritis. In recent years, RNAi drugs have been suggested to have the potential for repairing or regenerating the damaged articular cartilage. However, the effective delivery of small RNAs remains a

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Conflict of Interest

Dr. Yupeng Chen is co-founder of NanoDe Therapeutics.

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significant challenge. To overcome these obstacles, nanomaterial delivery systems including polymers, lipids, peptides, and oligonucleotide nanoparticles have been developed to enhance the effectiveness of RNAi drugs. Here, we review recent progress in using nanomaterials to deliver small RNAs for cartilage disease therapeutics.

In the future, nanomaterials can be specifically designed to form small-sized delivery vehicles with excellent penetration properties. RNAi therapeutics based on these nanomaterials will have great promise to treat cartilage diseases.

Keywords

Articular cartilage injury; Short interfering RNA; Nanomaterials; Delivery therapeutics

Introduction

Arthritis affects millions of people worldwide and is the most common cause of disability in the United States. This musculoskeletal condition causes significant impairment of mobility and has great social cost, especially in people older than age 65¹. Articular cartilage breakdown, as a hallmark of the arthritic disease, is difficult to repair and causes joint pain, stiffness, and loss of mobility². The most significant problems for drug-based treatment of arthritis have been related to drug administration and delivery. With oral medications, it is difficult to ensure sufficient enteral drug absorption allowing for transit of the medication into the bloodstream and ultimate drug penetration into the cartilage of the targeted joint. As a result, therapeutic effects in cell and matrix targets might not necessarily happen as desired³. Moreover, intra-venous (IV) administration carries risk of off-target malfunction from systemic delivery and may need to be reserved for patients with generalized, systemic diseases, as seen with current IV treatment regimens for rheumatoid arthritis and other autoimmune arthritis. The inherent risk of unintended side effects in other distant locations is a major reason why several potential osteoarthritis drugs have entered the stage of clinical practice⁴. The challenge of localized drug delivery to a single involved joint space (e.g. for treatment of cases of focal hip, knee, or shoulder osteoarthritis) likely means that precision guided injections, using ultrasound or fluoroscopic techniques, will be a necessary part of the treatment regimen.

In recent years, much research has been devoted to developing new strategies for repairing or regenerating the damaged osteoarticular tissue. RNA interference (RNAi) has been suggested to have the potential for implementation in targeted therapy in which the faulty gene can be edited by delivering its complementary Short Interfering RNA (siRNA) at the post-transcriptional stage^{5, 6}. The successful knock down of a specific gene by the delivered siRNA might slow or halt osteoarthritic diseases without side effects caused by chemical inhibitors⁷. However, cartilage siRNA delivery remains a challenging objective because cartilage is an avascular and very dense tissue with very low permeability⁸. Furthermore, RNA is prone to degradation by serum nucleases (such as RNase H and RNase A) due to an extra hydroxyl group in its phosphodiester backbone⁵. Therefore, successful delivery is the first and most crucial requirement for efficient RNAi therapy. There is sufficient need to develop carrier vehicles for siRNA delivery that ideally overcomes challenges of

internalization, degradation, and cell-specific targeting. Figure 1 demonstrated a general siRNA delivery process. Currently, the most commonly studied chemical carriers include cationic polymers and lipids, which have previously demonstrated potential in various forms of drug delivery^{9, 10}. Other biomolecules, such as cell-penetrating peptides and oligonucleotide nanoparticles have also been introduced as promising candidates for gene delivery.

1. Polymers

Various cationic polymers have been identified to have high potential for clinical applications. Due to their ability to encapsulate the negatively-charged nucleic acids, the resulting polyelectrolyte complexes not only allow the RNA to cross the cellular membrane, but also protect the therapeutic material from enzymatic degradation^{5, 11, 12}. Polymers are enticing delivery vehicles, because they are easily modifiable and can be constructed with specific physiological and physicochemical properties to optimize gene encapsulation, internalization, and cell-targeting^{11, 13–15}. Several cationic polymers have demonstrated gene binding and complexation capabilities both *in vitro* and *in vivo*, including Poly (L-lysine) (PLL), Polyethylenimine (PEI), and chitosan.

1.1. Poly (L-lysine) (PLL)

Poly (L-lysine) was one of the initial cationic polymers employed for gene delivery. Synthesized via polymerization of *N*-carboxy-anhydride of lysine, PLL is a linear polypeptide of repeating units of amino acid lysine, thereby being biodegradable^{12, 16}. When applied alone, PLL has limited clinical prospects due to its inadequate transfection efficiency, highlighting its tendency to rapidly associate with plasma proteins and to quickly be removed from circulation via excretion¹⁶. To improve the prospects of PLL-mediated gene delivery, various adaptations have been made to improve RNA delivery and uptake.

Chemical modifications, including addition of synthetic peptides or pH-sensitive entities to polymers, are often utilized to assist endosomal escape. PLL conjugated with cholic acid, a hydrophobic moiety, and poly(ethylene glycol) (PEG), demonstrated improved delivery potential along with reduced cytotoxicity¹⁷. These results are likely due to the masking effect of PEG upon the cationic charge density, thereby reducing toxicity and protecting against plasma enzymatic degradation. It is fairly easy to take advantage of the easily amendable nature of PLL in terms of structure, molecular size, and surface. Studies have even complexed PLL with silica nanoparticles or have used dendritic PLL to enhance siRNA uptake and transfection while reducing cytotoxic effects^{18, 19}.

1.2. Polyethylenimine (PEI)

PEI is one of the most potent gene carrier systems due to its ability to buffer at virtually any pH, thereby appropriately earning the term, “proton sponge”^{16, 20, 21}. This addresses the obstacle of endonuclease degradation because the pH buffer helps the carrier to bypass the endosomal barrier and avert lysosomal degradation, a critical step in achieving successful gene transfection¹¹. PEI has been identified as a promising gene delivery agent for systemic application, capable of complexation with siRNA to induce c-erbB2/neu (HER-2) down-

regulation²². Unfortunately, one of the disadvantages of PEI is its limited degradability - leading to high levels of cytotoxicity that can interfere with transfection activity^{23, 24}. Studies have suggested that this toxicity depends on properties of PEI itself, including molecular weight and structure. Although cytotoxicity decreases with increasing linearity of PEI, transfection efficiency also suffers^{11, 25}. Consequently, researchers have designed chemical modifications to PEI by conjugating a variety of degradable cross-linkers to help facilitate intracellular degradation either by hydrolysis, enzymatic function, or cytosolic reductive action by glutathione¹¹. Furthermore, although gene transfer efficiency increases for higher molecular weight varieties of PEI, cytotoxicity is also enhanced with larger polymer size. Therefore, a reasonable approach to PEI modification is to facilitate RNA delivery via low cytotoxicity, low molecular weight (LMW) PEI linked to biodegradable conjugates of suitable higher molecular weights²⁶.

Exploiting the modifiable nature of the polymers allows researchers to explore cell-targeting or biodegradable ligands to enhance the delivery mechanism by producing a more sterically stabilized carrier vehicle. Similar to PLL modifications, charge neutralizing agents such as poly(ethylene glycol) (PEG) have been conjugated to PEI and have exhibited improved transfection with lower toxicity levels compared to non-PEGylated PEI polyplexes^{16, 26}. These modifications can mitigate the excessive net positive charge of cationic polymers that make them more capable of associating with anionic serum proteins. Other approaches have included linking degradable polyglutamic acid derivative backbones to PEI or crosslinking with disulfide bonds, both of which exhibited increased degradability and target gene down-regulation^{25, 26}.

Interleukin (IL)-15 plays an important role in the pathogenesis of rheumatoid arthritis (RA). Systemic knockdown of IL-15 receptor (IL-15R) might be an effective way for a reduction in inflammation at local sites. The IL-2/15R β siRNA, which can target the β chain of IL-15R and IL-2 were formulated with the commercial cationic polymer, in vivo-jetPEI, to be PEI-complexed siRNA nanoparticles. The nanoparticles can easily accumulate in arthritic paws of rats and are then taken up by immunocytes. IL-2/15R β siRNA polyplexes exhibit an inhibitory effect on disease progression of RA assessed through swelling and arthritis scores indicating successful delivery of IL-2/15R β siRNA to the arthritic joints²⁷.

1.3. Chitosan

First implicated in gene delivery in 1995 by Mumper et al., chitosan is a biocompatible and resorbable cationic aminopolysaccharide of glucosamine units¹⁶. As high toxicity exhibited by cationic polymers has limited their clinical application²⁸, greater interest has been focused on natural biodegradable polymers like chitosan. It was not until 2006 that chitosan was used for in vitro delivery of siRNA, which again emphasized the importance of proper chitosan-siRNA composition and formulation techniques, such as simple complexation, siRNA entrapment via ionic gelation, or siRNA adsorption onto chitosan nanoparticles^{29, 30}. Additional studies have identified a variety of critical elements influencing physicochemical properties and transfection efficiency, such as composition and formulation of the chitosan-nucleic acid³¹.

As with other polymers mentioned, chitosan is also subject to modifications aimed at improving therapeutic utility. The therapeutic application of chitosan is limited due to its insolubility in neutral pH; however, chitosan oligomers—short chains of chitosan produced by depolymerization of chitosan with nitrous acid—have demonstrated favorable pharmaceutical characteristics including solubility in water and reduced viscosity³¹. Another modification to yield improved transfection efficiency is to use trimethylated chitosan in which primary amine groups are replaced with quaternary amino groups. By introducing a quaternization degree, solubility significantly increased with increased transfection efficiency in fibroblast cell lines³¹. Shi et al developed another modified chitosan gene nanocarrier which consists of folic acid, diethylethylamine and polyethylene glycol. The nanocarrier was utilized to deliver siRNA capable of silencing tumor necrosis factor- α (TNF α), a pro-inflammatory cytokine playing a role in the pathophysiology of rheumatoid arthritis. This novel nanocarrier not only provides good protection for the siRNA against nuclease destruction but also facilitates siRNA uptake by folate ligands. The successful siRNA delivery can control inflammation as well as bone and cartilage destruction in rheumatoid arthritis²⁹.

2. Lipids

2.1. Cationic lipids

Cationic lipids have been utilized in gene therapy since (*N*-[1-(2,3,-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride) (DOTMA) was first introduced in 1987 by Felgner et al³². Identified as one of the more promising non-viral systems for gene therapy, cationic lipids have previously been implicated as delivery vectors for DNA-based drugs. Liposome-based carriers are attractive for gene delivery, as they can be formulated to about 100 nm in size and have generally inert metabolic products^{33–35}. Additionally, liposomes are able to protect encapsulated oligonucleotides from nuclease degradation and renal clearance³⁶. Cationic lipids execute three important roles in siRNA delivery. First, by electrostatic association, cationic lipids encapsulate and condense siRNA. Next, the net positive charge of the lipid-based nanoparticle enhances interaction with the negatively-charged cell surfaces, thus promoting internalization. Finally, the resulting destabilized endosomal membrane facilitates cytoplasmic siRNA delivery^{33, 37}. Unlike polymers, cationic lipids destabilize endosomal membrane by electrostatically interacting with anionic lipids to form ion pairs, forming an inverted hexagonal (H_{II}) phase. The H_{II} phase is an intermediate structure that forms during the fusion between the cationic and anionic lipids. This step is not only crucial for membrane destabilization, but also for nanoparticle disassembly resulting in cytoplasmic cargo delivery³³. In aqueous solutions, DOTMA aggregates spontaneously with anionic nucleic acid, forming gene-encapsulated liposomes.

Various cationic lipids have been developed, including an analog of DOTMA known as DOTAP (1,2-diacyl-3-trimethylammonium propane), a family of lipids containing ester as linker bonds. DOTAP-based liposomes have been applied *in vivo* to address metabolic skeletal disorders by silencing bone-formation inhibitory genes in osteogenic lineage cells. With the conjugation of a targeting moiety consisting of 6 repetitive sequences of aspartate, serine, serine (AspSerSer)₆, DOTAP-mediated siRNA delivery demonstrated high binding

affinity to the bone-forming surface, along with down-regulation of its target gene, *Plekho1*, thus providing a potential point of access to the clinical translation of RNAi-based arthritis therapy⁸.

Another lipid-mediated gene delivery of interest is via stable nucleic acid lipid particle (SNALP). Of the lipid nanoparticle (LNP) family, SNALP is a popularly utilized approach to *in vivo* delivery of siRNA³⁸. SNALP favorably generates uniformly sized nanoparticles with high siRNA encapsulation efficiency, and has progressed to clinical trials since achieving gene silencing in nonhuman primates³⁹. Recently, a novel positively charged LNP-siRNA delivery system was developed by mixing a lipid mixture ethanol stream with a siRNA aqueous stream. The system was used to knock down the cartilage-specific gene Indian Hedgehog (*Ihh*) *in vivo*. The increased activity of *Ihh* has been considered closely related to osteoarthritis progression in humans. This study demonstrated that siRNA could be delivered by LNPs into the knee joint to knock down *Ihh* and inhibit cartilage degeneration⁷.

Although laboratory use of commercial cationic lipids such as lipofectin, lipofectamine, or transfectam is well-established, their use may not be effective for clinical application. Newer lipid-based gene delivery strategies have focused on polyvalent cationic head groups containing long carbon chains connected by either glycosidic groups, diethers, or disulfide groups¹⁶. The performance of various lipid-mediated siRNA delivery has also been investigated to identify the structural motifs that could achieve high levels of knockdown, and many of these elements focused on the amines per head unit and acyl chain properties⁴⁰.

2.2. Shielding lipids

As with polymers, PEGylation serves multiple beneficial purposes for liposomal RNA delivery, mainly prolonging circulation half-life by reducing opsonization⁴¹. Steric hindrance due to the high chain motility of PEG limits adsorption of proteins, such as opsonins. Subsequently, activation of the complement pathway is avoided, as is the recognition and clearance of the liposome by macrophages⁴². However, the shielding effect mediated by PEG has shown to hinder membrane fusion between the liposome and endosomal membrane, reducing cellular uptake by target cells and decreasing efficacy *in vitro* and *in vivo*⁴³. Two strategies have been introduced to improving PEGylated nanoparticle efficacy. One involves conjugation of PEG to the liposome by acid-sensitive bonds, such as oxime linkers, which were shown to have improved siRNA release at pH 5.5 and enhanced gene silencing *in vivo*⁴⁴. Another method is to utilize pH-sensitive modified PEG linked to liposomes via ionic interactions. Although the modified PEG (covalently linked to HEMA-histidine-methacrylic acid) has a net negative charge at physiological pH, the methacrylic acid becomes protonated in the endosome, thus changing the net charge of PEG to positive. The reversion of the net charge causes the release of PEG from the lipid core, exposing the cationic liposomal membrane and allowing fusion with the endosome⁴⁵.

3. Peptides

3.1. Cell-penetrating peptides

Cell-penetrating peptides (CPPs) have been utilized as delivery vehicles for various molecular cargos, including fluorescent dyes, proteins, and oligonucleotides. With the discovery that the full-length HIV-1 TAT protein alone was able to penetrate the cell membrane and induce *trans*-activation of the HIV-1 promoter, the peptide fragment conferring cell permeability was identified⁴⁶. The Tat peptide, as it is known, is characterized by a high density of basic residues (e.g. lysine or arginine) that are proposed to mediate cellular uptake by interacting with the anionic surface of the plasma membrane and enhancing peptide internalization^{47–49}. Since this initial observation, there have been more CPP-peptides identified, such as the MPG, a new peptide-based gene delivery system, and synthetic chimeric peptides such as model amphipathic peptide (MAP)^{50, 51}.

Two main approaches to CPP-mediated siRNA delivery have been identified: non-covalent CPP/siRNA complexation and covalent attachment of CPP to siRNA via disulfide bonds. The non-covalent complexation utilizes the dense cationic charge of CPPs to condense siRNAs into nanoparticles with a net positive charge. The covalent linkage of CPP to siRNA is the preferred method, as this yields small, monomeric CPP/siRNA molecules that can evade various *in vivo* complications associated with cationic condensation of nucleic acids^{48, 52–54}. However, covalently linking the negatively charged siRNA to CPP neutralizes the overall charge of the CPP/siRNA complex, jeopardizing internalization by reducing ability to bind to the cell surface.

CPP-mediated RNA delivery in mammalian cells was achieved with the MPG-peptide-based system. The MPG peptide is a fusion of the HIV-1 gp41 peptide domain with the nuclear localization signal (NLS) of the SV40 large T antigen^{50, 55}. Through non-covalent complexes with nucleic acids, the MPG peptide demonstrated plasmid DNA delivery via endosomal pathway-independent cell entry with intact nuclear targeting. By disrupting the NLS through a point mutation, nuclear delivery is impaired; therefore, MPG is applicable for cytoplasmic delivery of siRNA. Application of the MPG-siRNA system elicited rapid siRNA release with down-regulation of the target mRNA⁴⁵.

Overall, CPP's are a promising class of delivery vectors due to their ability to overcome notable barriers to delivery, including condensing nucleic acids into compact particles, targeting specific cell receptors, and promoting endosomal escape. However, the mechanism of entry is still yet to be fully elucidated, as they may differ based on specific conditions such as the type of CPP, type of cargo, and the type of cell^{47, 55}.

4. Oligonucleotide Nanoparticles

Three dimensional oligonucleotide nanostructures have been explored for encapsulation of cargo, ranging from imaging to therapeutic delivery applications^{56–59}. Oligonucleotide nanoparticles (ONPs) formed via programmable self-assembly of short DNA and RNA fragments with therapeutic siRNA have shown to induce gene-silencing activity. Unlike conventional delivery nanoparticles (NP's) that are heterogeneous in size, composition, and

surface chemistry resulting in suboptimal performance and even cytotoxicity, ONPs have a defined size, structure, and stoichiometry^{60, 61}. A self-assembled tetrahedron composed of six DNA strands forms six edges of 30 base pairs long, with a centrally located nick that is complementary to the siRNA overhang. Therefore, six siRNA molecules can be bound per nanoparticle. Other than natural nucleic acids, modified molecules were also used to build nanotubes and other nanostructures for therapeutic delivery^{62–67}. As shown in Figure 2, Janus base molecules were synthesized mimicking DNA base pairs. Six Janus bases can self-assemble into a rosette plane via hydrogen bonds and these rosette planes can further stack into therapeutic delivery ONPs via π - π interactions^{62–64}. Moreover, the nucleic acid composition of ONPs can be easily manipulated, allowing for specific hybridization of siRNA sequences. Folate-modified ONPs have demonstrated gene knockdown in mice expressing folate-receptor positive tumor xenografts⁶⁰. However, clinical application of DNA NPs can be limited by their relative structural and functional simplicity; therefore, other RNA-based nanostructures have been developed. Bacteriophage phi29 packaging RNA (pRNA) is widely used for RNA nanoparticle assembly due to its structural features. These RNA NPs can then be functionalized by siRNA. Different oligomers can be formed to include interlocking loops or palindromic sequences to control unpredictable off-target effects. Additionally, RNA NPs are already highly soluble and not prone to aggregation, and do not need to be linked to PEG or serum albumins⁶¹.

Limitations

While the many siRNA options listed within this review have great clinical promise, the clinical applications of DNA- or RNA-based delivery vehicles are currently limited for a number of reasons. An ideal method of precise drug delivery to affected joint(s) limits the capability of various therapeutic options, and the potential risk of systemic side effects is of clinical concern for any type of oral or IV medication at this time. In addition, for any drug utilized, there are current gaps in understanding the pharmacokinetics and toxicology associated with clearance, plasma kinetics, and systemic distribution^{68, 69}. Furthermore, sensitivity to RNase degradation and possible dissociation due to non-covalent linkage of self-assembled RNA quaternary structure, which can be improved by RNA ribose ring modification, still need to be further elucidated⁶¹.

5. Discussion and Conclusion

Physical impairment due to arthritis is common, debilitating, and expensive⁷⁰. In many cases, therapeutic treatment is imperfect because it is difficult to achieve meaningful drug penetration and transport into cartilage. Gene-based therapy, specifically RNAi, has been implicated in therapeutic applications due to its versatility and specificity. In the context of arthritic disease, RNAi might improve repair and regeneration at sites of injury by enabling the local, sustained, and potentially regulated expression of therapeutic gene products, including morphogens, growth factors, and anti-inflammatory proteins⁷¹. In addition, enhanced capability of drug penetration into cartilage to facilitate stimulation of chondrocytes through utilizing targeted methods will also have the potential to usher in a meaningful new era of “Regenerative Medicine” and “Cartilage Restoration”. However, to achieve the clinical potential of RNAi, delivery materials are required to transport siRNA

into the cells of target tissues considering the easily enzymatically degraded of the siRNA. SiRNA-based treatment against cartilage and joint diseases is still very new. Currently, there was no clinical trial on any of these therapeutics. With the development and perfection of RNA delivery technologies, we believe siRNA will successfully translate into clinical settings in near future.

In this review, we summarized the commonly used delivery vehicles for naked siRNA, including cationic polymers, cationic lipids, peptides, and oligonucleotide nanoparticles. Significant improvements to these nanomaterials, namely chemical modifications, have been implemented to address barriers in siRNA delivery to joints. By successful delivery of siRNA into the joints, decreasing the progression of inflammation and cartilage degeneration can be achieved. As we presented in the review, the siRNA-based therapeutics provide great promise for arthritic disease therapy, but the clinical application of these advanced scientific concepts remains elusive. Further translational research will help to set the stage for longitudinal assessment of the effects of these various classes of medication. Once the drug delivery, cartilage membrane penetration, and cellular modification steps have been elucidated, this unique class of medications have a potentially large impact on the lessening the future impairment of patients due to musculoskeletal disease.

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Figure 1.
A schematic demonstration of delivery vehicles and their cell targeting and uptake processes.

Figure 2.
The rosette plane (left) and the nanotube vehicle (right) formed by Janus base molecules.