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Photoinduced RNA Interference

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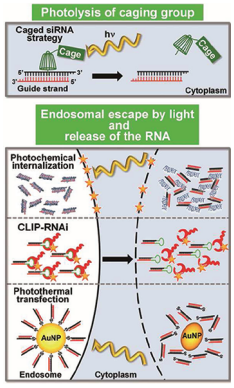
CONSPECTUS

Because RNA interference (RNAi) can be applied to any gene, this technique has been widely used for studying gene functions. In addition, many researchers are attempting to use RNAi technology in RNAi-based therapies. However, several challenging and controversial issues have arisen during the widespread application of RNAi including target gene specificity, target cell specificity, and spatiotemporal control of gene silencing. To address these issues, several groups have utilized photochemistry to control the RNA release, both spatially and temporally.



In this Account, we focus on recent studies using photocleavable protecting groups, photosensitizers, Hand gold nanopartides for photoinduced RNAi. In 2005 the first report of photoinduced RNAi used a caged short interfering RNA (siRNA), an siRNA carrying a photocleavable protecting group. Caging groups block the bioactivities of target molecules, but allow for complete recovery of these functions via photoactivation. However, some RNAi activity can occur in these caged siRNAs, so it will be necessary to decrease this "leakage" and raise the RNAi activity restored after irradiation. This technique also uses UV light around 350 nm, which is cytotoxic, but in the near future we expect that it will be possible to use visible and near-infrared light

We also examine the application of photochemical internalization (PCI) to RNAi technology, which involves a combination of photosensitizers and light. Instead of inducing RNAi using light, the strategy behind this method was to enhance RNAi using RNA carriers. Many wellknown RNA carriers deliver siRNAs into cells by endocytosis. The siRNAs are trapped in endocytic vesicles and have to be released into the cytoplasm in order to express their activity. To achieve the endosomal escape of siRNAs, PCI technology employed photosensitizers to generate light-dependent reactive oxygen species (ROS) that disrupted the endocytic vesicles. In most studies, RNAi-mediated knockdown of the target gene was detected even without PCI. Recently, a polymer capable of trapping the siRNA in endocytic vesicles controlled RNAi almost entirely by light. CLIP-RNAi uses photosensitizing carrier proteins that can be activated over a wide range of visible light wavelengths. With this method RNA carrier/siRNA complexes are completely trapped within endosomes, and RNAi is controlled strictly by light. Such precise, light-dependent control will open up new possibilities for cellular and molecular biology and therapy.

Most recently, gold nanoparticles (AuNPs) conjugated to siRNA have provided temporal and spatial control of RNAi. The light-dependent melting of AuNPs accompanied by a shape transformation induces the release of thiolated siRNAs from AuNPs. In this method, the unique optical properties of the AuNP enable deep penetration of the excitation light into tissues at nearinfrared wavelengths.

The development of photoinduced RNAi technology will lead to novel insights into gene functions and selective drug delivery, and many other scientific fields will continue to influence its progress.

Introduction

RNA interference (RNAi) is a powerful mechanism of gene silencing, in which a target mRNA is specifically degraded by a short interfering RNA (siRNA).[1] Since complete genome sequences have been decoded in various species, RNAi is being widely explored as a therapeutic strategy for intractable diseases, including cancer, neurodegenerative diseases, and viral infections, and as a tool for functional analysis.[2] Compared to conventional gene silencing techniques using ribozymes or antisense and antigene oligonucleotides, RNAi is a convenient and general-purpose technique. However, there is room for improvement in target gene specificity, target cell specificity, and spatiotemporal control of gene silencing. One major challenge for current RNA delivery is to control the release of the interfering RNA both spatially and temporally, because local delivery of siRNA, that is, reaching target cells within a target tissue or organ, is required to decrease the side effects

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associated with RNAi therapeutics and gene function studies.

Recently, several groups have concentrated on the use of photochemistry as a potent inducer of RNAi. Light is a more favorable inducer for finely controlling RNAi on a microscale than chemical inducers and is safer than electricity, which is used as an inducer in electroporation methods. In this Account, we focus on recent topics of interest in the photoinduced RNAi field, including the caged siRNA strategy, photochemical internalization (PCI), photothermal transfection, and a method recently developed in our laboratory that we have called CLIP-RNAi (CPP-Linked RBP-mediated RNA Internalization and Photo-induced RNAi).

Caged siRNA Strategy

A caged biomolecule is a molecule carrying a photocleavable protecting group that blocks the native biochemical or biological activity of the molecule. Caged biomolecules can be activated by light,[3] and the use of many caged compounds, such as ATP, glutamate, and nucleic acids, in the control of bioactivity have been reported to date.[4] In addition, a number of caging groups that block the bioactivities of target molecules and allow complete recovery of their functions following photoactivation, have also been reported.[4] Due to limited space in this Account, those readers interested in caged biomolecules should refer to a recent review.[4]

The first report of a caged siRNA, which was also the first report of photoinduced RNAi, was described by Shah et al. in 2005.[5] This group used 1-(4, 5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) as the photolabile caging group, which protected phosphate groups of the RNA backbone and resulted in a 3% caging efficiency (1.4 photocaging groups per siRNA duplex) (Figure 1). The use of siRNA-mediated RNAi involves multiple steps: association of the siRNA with the RNA-induced silencing complex (RISC), cleavage and unwinding of the sense strand to activate the RISC, RNA-RISC guidance to the complementary mRNA and destruction of the targeted mRNA.[6] Therefore, Shah et al. formulated an approach in which the covalent attachment of the caging group, DMNPE, to an siRNA duplex sterically blocked the association of the siRNA with the RISC, resulting in RNAi inhibition. Lipofectamine was used to transfect the caged anti-GFP siRNA into HeLa cells, transiently expressing GFP, and the RNAi efficiencies were examined by evaluating GFP silencing in cells with or without photoirradiation (Blak-Ray fluorescent UV lamp, 15 W, 12 min). As expected, when the cells were treated with the caged siRNA,

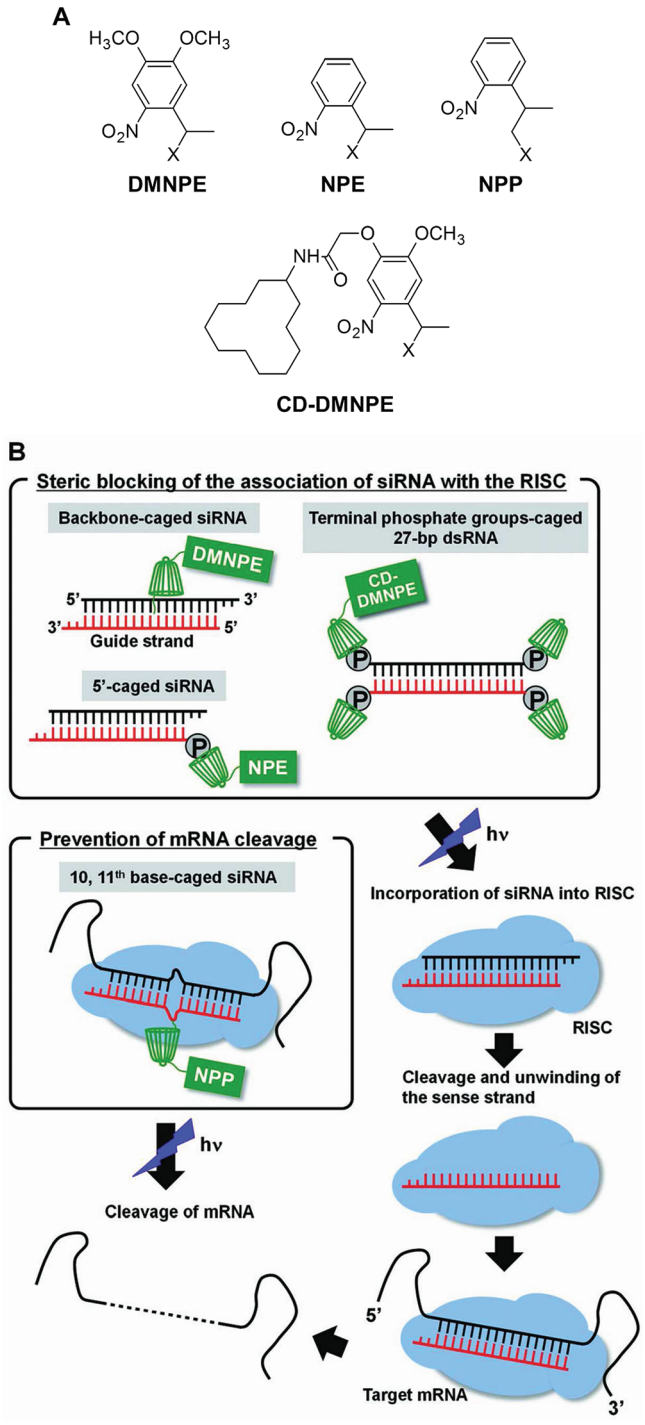

FIGURE 1. (A) Structure of caging groups for siRNA. DMNPE (1-(4,5-dimethoxy-2-nitrophenyl)ethyl), NPE (1-(2-nitrophenyl)ethyl), NPP (2-(2-nitrophenyl)propyl), and CD-DMNPE (cyclo-dodecyl DMNPE). (X = siRNA or dsRNA). (B) Schemes of caged siRNA strategies. Backbone-caging of siRNA with DMNPE groups and 5′-caging of siRNA with an NPE group sterically block association of the siRNA with RISC. Tenth or eleventh base-caging of the siRNA with an NPP group prevents mRNA cleavage. Caging of dsRNA terminal phosphate groups prevents binding with Dicer and/or nucleases due to steric hindrance, which thus prevents generation of siRNA that can associate with RISC. See details in text.

GFP silencing was induced in a light-dependent manner; in addition, the RNAi efficiency induced by photolysis of the caged siRNA was equal to that induced by uncaged siRNA (a decrease of approximately 70% in GFP expression compared to control cells without the siRNA). The authors demonstrated that there was no phototoxicity associated with this method and no toxicity resulting from release of the DMNPE group. However, it is important to note that GFP silencing was observed in cells treated with the caged siRNA even without irradiation (40% "leakage" activity), indicating that inactivation of the caged siRNA was incomplete.

Accordingly, several efforts to reduce this leakage activity have been reported. Nguyen et al. attached a 1-(2-nitrophenyl)ethyl (NPE) group, as the caging group, to the 5′ terminal phosphate of the siRNA guide strand,[7] which is thought to be critical for incorporation of the siRNA into RISC (Figure 1).[8] The authors demonstrated that NPE-caged siRNAs induced glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a light-dependent manner (345−385 nm, 2 J/cm^2, approximately 70% RNAi efficiency using Lipofectamine 2000, and approximately 80% RNAi efficiency using the MPG peptide (27 aa),[9] which is a bipartite amphipathic peptide derived from the fusion of HIV-1 gp41 and the nuclear localization sequence of the SV40 large T antigen) and that highly purified caged siRNAs were important for successful inhibition of the leakage activity. Increased knockdown of GAPDH prior to light activation correlated with a decrease in caging efficiency. The purity of the caged siRNA on a routine basis (>95%) elicited a 30 to 40% knockdown without light. Nevertheless, other groups interpreted similar results as indicating that the RISC has a fraction of the ability to interact with caged siRNAs.[10] In either case, controversial leakage of the activity of caged siRNAs remained.

In 2007, Mikat and Heckel reported the use of photolabile modifications (2-(2-nitrophenyl)propyl (NPP) groups) attached to the base moiety of deoxyguanosine or deoxythymidine, which were introduced into the antisense strand of the siRNA (Figure 1).[11] It has been shown that a modification in the central part of the antisense strand leads to a bulge in the siRNA:mRNA duplex, resulting in complete inhibition of RNAi.[8,12] The replacement of uridine by NPP-protected deoxythymidine at position 10 of an anti-EGFP siRNA antisense strand resulted in photoinducible RNAi with irradiation at 2.88 J/cm^2 (λ_{max} = 366 nm), whose knockdown efficiency and "leakage" activity were about 75% and <10%, respectively, after 24 h of incubation. Both the RNAi efficiency and inhibition of the "leakage" activity in this method are superior to the other methods mentioned above, although the "leakage" RNAi activity increased to around 40% after 42 h incubation with the caged siRNA (∼20% after 28 h incubation), perhaps due to cleavage of the caging group by the cell's oligonucleotide repair system, or simply as a consequence of the chemical environment within the cell.

To achieve full inactivation, multiple caging group attachment sites to siRNAs might be required, and in fact, it has been shown that increasing the number of attached caging groups raises the efficiency by which the RNAi activity is blocked prior to irradiation.[5,11] However, these reports showed that the restoration of RNAi activity after irradiation was prevented by multiple caging groups, despite the improvement in blocking efficiency. Hence, improving the balance between the drawbacks and the benefits of various refinements may be critical for the expanded use of caged siRNAs.

Most recently, Jain et al. proposed that the incomplete block of RNAi prior to irradiation might be because (1) the caging groups do not completely hinder binding to Dicer, which cleaves double-stranded RNAs (dsRNAs) to generate siRNAs, or (2) the caging groups can be removed by nucleases that remove terminal residues from the caged RNA.[13] The authors designed a new bulky photolabile caging group, cyclo-dodecyl DMNPE (CD-DMNPE), and attached it to a blunt-ended 27-bp dsRNA to prevent binding with Dicer and/or nucleases through steric hindrance (Figure 1). In contrast to the DMNPE group used previously,[5] the siRNA, terminally modified with four CD-DMNPE groups, controlled target gene expression perfectly in a light-dependent manner, with an RNAi efficiency and "leakage" activity of 89% and 3%, respectively.

However, the widespread use of caging groups is also limited by the optical wavelength for deprotection, which must be around 350 nm.[14] Although photolabile caging groups, which can be deprotected efficiently by irradiation at 350 to 400 nm, have been developed, near-ultraviolet and visible light has poor tissue penetration due to scattering and light absorption by hemoglobin and biomolecules.[15] Additionally, infrared light (>900 nm) is absorbed by water. Therefore, near-infrared (NIR) light at around 650 to 900 nm, which can penetrate deep into the tissue, is useful in biological systems.

The use of two-photon excitation systems provides a possible solution to this problem.[16] In principle, a caging group that normally absorbs ultraviolet light (∼350 nm) can

also be excited by two red photons (~700 nm) when they reach the caging group at the same time.[17] In this case, the light penetrates deeper into the tissues and only the caging groups near the focal point of the focused NIR beam are excited. However, the two-photon excitation system requires high excitation power density and special equipment (focused NIR light source).

Recent years have seen exciting developments in the application of upconverting nanoparticles (UCNPs) to caging technology.[18] UCNPs are luminescent nanomaterials that convert near-infrared excitation into visible emissions through lanthanide doping.[19] Carling et al. used $NaYF_4$ nanoparticles doped with the lanthanides, Tm^{3+} and Yb^{3+} ($NaYF_4$:TmYb), in combination with a caging group (3′,5′-di(carboxymethoxy)benzoin). $NaYF_4$:TmYb nanoparticles are some of the best UCNPs because they convert NIR (980 nm) light into UV, visible, and NIR light.[20] Indeed, NIR irradiation (980 nm, 550 W/cm^2) of 3′,5′-di(carboxymethoxy)benzoin-caged $NaYF_4$:TmYb nanoparticles induced photolysis of the 3′,5′-di(carboxymethoxy)benzoin group as efficiently as UV irradiation (290 nm). Thus, photolysis of the caging group, which induced the photorelease of acetic acid, was achieved using a combination of NIR light and upconverting nanoparticles. A recent report has described the use of biocompatible and nontoxic nanoparticles for *in vitro* and *in vivo* applications based on lanthanide-doped $NaYF_4$ nanoparticles.[21] However, although this UCNP-based system overcomes the fundamental problem of the light source commonly associated with caged siRNA-mediated gene silencing, the system has not yet been applied to photoinduced RNAi. Further refinements will be necessary before their use can be extended to biomolecules such as siRNAs (Figure 2).

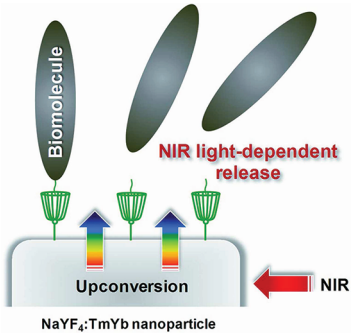

FIGURE 2. Diagram showing photorelease of biomolecules by NIR light using an upconverting nanoparticle ($NaYF_4$:TmYb). $NaYF_4$:TmYb nanoparticles can convert NIR (980 nm) light into UV, visible, and NIR light. Photolysis of caging groups tethered to nanoparticles is induced by NIR exposure.

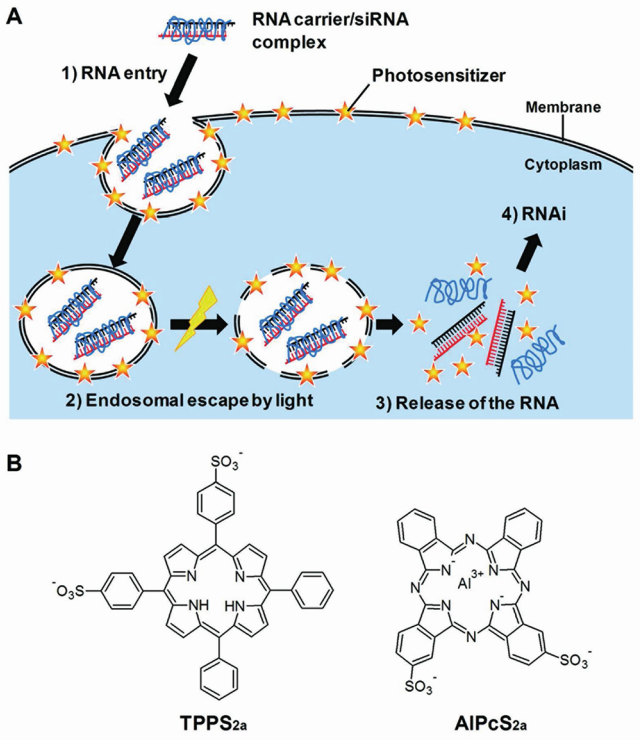

FIGURE 3. (A) Structure of photosensitizers used in PCI. $TPPS_{2a}$ (tetraphenylporphine disulfonate) and $AlPcS_{2a}$ (aluminum phthalocyanine disulfonate). (B) Scheme of the PCI strategy for photoinduced RNAi. Photosensitizers are first incorporated into endocytic vesicles with siRNAs; next, endocytic membranes are disrupted by the ROS generation following light-activation of photosensitizers; finally, siRNAs are released into the cytosol and RNAi is induced.

Photochemical Internalization (PCI)

PCI is a site-specific intracellular delivery technology, which ruptures the membrane of endocytic vesicles inducing cytosolic release of therapeutic molecules and biomolecules by photostimulation.[22] Strategic processes in this technology include (1) incorporation of photosensitizers with target molecules in endocytic vesicles, (2) the generation of ROS as a result of light-activation of the photosensitizers, and (3) subsequent disruption of the membranes of endocytic vesicles and release of target molecules to the cytosol (Figure 3A). Given the subsequent RISC loading process, the RNA has to be, at least partially, released from the carrier. The principle behind PCI is similar to that of photodynamic therapy (PDT), which is a conventional approach using a combination of photosensitizers and light.[23] In the case of PDT, nuclei, mitochondria, lysosomes, and the plasma membrane have all been evaluated as potential PDT targets; thus, specific localization of photosensitizers is not required, and

photochemical reactions completely eradicate photoirradiated cells. On the contrary, in the case of PCI, photosensitizers should accumulate specifically in endocytic membranes and photochemical reactions should occur ideally only in the membranes of endocytic vesicles.[24] In fact, the internalized fluorescently labeled siRNA showed a punctate cytoplasmic distribution that colocalized with endosome markers. When the cells were exposed to light, a change in fluorescence distribution throughout the cytoplasm, from punctate to diffuse, was observed suggesting that disruption of the endosomes was induced by light.[25] Tetraphenylporphine disulfonate ($TPPS_{2a}$) and aluminum phthalocyanine disulfonate ($AlPcS_{2a}$) have been reported as the most useful photosensitizers for PCI (Figure 3B).[26] Both compounds possess an amphiphilic character, which allows them to bind to the plasma membrane and localize to the membranes of endocytic vesicles via endocytosis.

The first application of PCI in RNAi used $TPPS_{2a}$ as a photosensitizer combined with an antiepidermal growth factor receptor (EGFR) siRNA.[25] Complexes of 160 nM anti-EGFR siRNA and Lipofectamine (anti-EGFR siRNA/LF) were prepared and introduced into cells. The cells were incubated further with, or without, 0.4 μg/mL $TPPS_{2a}$. EGFR knockdown was detected in cells treated with the anti-EGFR siRNA/LF and $TPPS_{2a}$ (375−450 nm, 0.78 J/cm^2, 70 to 80% RNAi efficiency), and the efficiencies depended on both the concentration of $TPPS_{2a}$ and the light exposure time. However, even without PCI, EGFR knockdown (20% efficiency) was observed in cells treated with the siRNA/LF complexes alone. In another study by the same group, PCI-mediated gene silencing was also examined in mouse models.[27] A431 tumor cells from xenografted mice, which express a high level of EGFR protein, were treated with a combination of anti-EGFR siRNA/LF and PCI. The authors selected the photosensitizer $AlPcS_{2a}$, which has its major absorbance at 670 nm, a wavelength capable of penetrating the skin and reaching target tissues (the absorbance for $TPPS_{2a}$ is 435 nm). Treatment of the murine tumors with a combination of anti-EGFR siRNA/LF and $AlPcS_{2a}$ followed by photostimulation (670 nm, 75 J/cm^2) induced a decrease of about 80% in EGFR expression when compared with the untreated mice. However, 30% knockdown of EGFR was also detected in murine tumors treated with anti-EGFR siRNA/LF alone. Thus, in this case, PCI enhanced RNAi using a siRNA−lipid complex rather than inducing RNAi by light.

Subsequently, Boe et al. reported a PCI-dependent silencing effect when using the cationic lipid, jetSI (or jetSI-ENDO), and polyethylenimine (PEI) under optimized conditions.[28] In both cases, however, "leakage" activities using jetSI and PEI were estimated at ∼30% and ∼20%, respectively. More recently, in another study by Boe and co-workers, RNAi was controlled almost entirely by light using β-cyclodextrin-containing polymers based on six methylene units (β-6CDP),[29] which were synthesized for the delivery of nucleic acids. β-6CDP does not have a strong buffering capacity, which is advantageous for endosomal escape.[30] Boe et al. investigated whether siRNA transfection, using β-6CDP with $TPPS_{2a}$, induced RNAi-mediated gene silencing in a light-dependent manner.[29] For PCI-induced RNAi, the human S100A4 protein in OHS cells was used as the target gene. In the cells treated with the anti-S100A4 siRNA/β-6CDP complex, $TPPS_{2a}$, and light exposure of 280 J/cm^2, the S100A4 silencing efficiency was nearly 90% compared with the untreated control, and cell viability was unaffected by the treatment. Importantly, PCI in combination with β-6CDP successfully minimized "leakage" activity to between 0% and 10%, which was much lower than reported previously for PCI-induced RNAi using 25 kDa B-PEI, jetSI, or LF as a carrier. These results implied that the siRNA/β-6CDP complexes were completely trapped within the endosomes.

In this method, depending on the intensity of the irradiation, nonspecific suppression of gene expression was also induced,[27,29] possibly due to a PDT-effect.[27] Therefore, fine-tuning of the irradiation intensity is required for specific photoinduced RNAi. PCI technology is promising in that it has the potential of being able to deliver pharmaceutical agents in a highly specific manner, and is in fact undergoing phase I/II clinical trials (U.S. National Institute of Health clinical trials Web site).

CLIP-RNAi

CLIP-RNAi was developed recently in our laboratory as a photoinducible RNAi method using photosensitizing carrier molecules.[31] CLIP-RNAi is an attractive technique that regulates specific gene expression by photostimulation. The photosensitizing carrier used in this method consists of a cell-penetrating peptide (CPP), an RNA-binding protein (RBP) and a fluorescent dye as a photosensitizer (Figure 4). Using the synthetic conjugate molecule (CPP-RBP-dye) as an RNA carrier, we succeeded in the specific delivery of an shRNA containing an RBP binding sequence in its loop region.[31b]

The attachment of a CPP to a cargo molecule helps to improve the intracellular delivery of cargo molecules such as low-molecular compounds, peptides, and proteins.[32] One well-known CPP is the 11-amino acid positively charged TAT

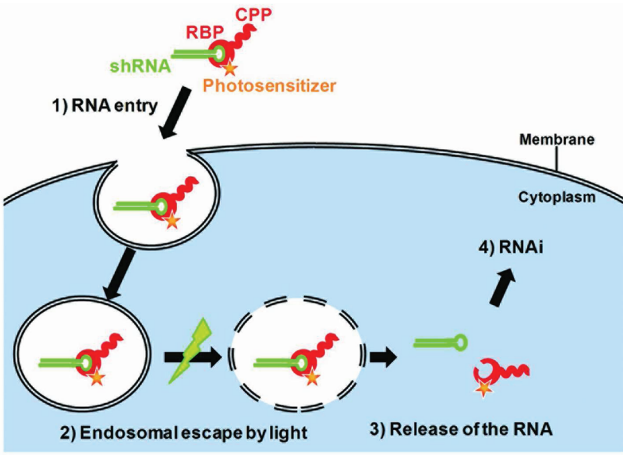

FIGURE 4. Scheme of CLIP-RNAi strategy. CPP-RBP is a fusion protein between a cell-penetrating peptide (CPP) and an RNA-binding protein (RBP). The CPP-RBP attached to a fluorescent dye as a photosensitizer (CPP-RBP-dye) can undertake the specific delivery of an shRNA containing an RBP binding sequence in its loop region. The complexes formed between the CPP-RBP-dye and shRNA are internalized by cells via an endocytotic pathway. CPP-RBP-dye/shRNA complexes trapped in endosomes are released into the cytosol in a light-dependent manner.

(Trans-Activator or Transcription) peptide, which the human immunodeficiency virus type 1 TAT protein uses to enter a cell across the cytoplasmic membrane.[33] The first RNA carrier protein (CPP-RBP) designed by our group was TatU1A, in which the RNA-binding domain of the U1 small nuclear ribonucleoprotein A (U1A) is fused to the TAT peptide as the CPP.[31b] Some reports have suggested that macropinocytosis is the main pathway for CPP uptake, especially when CPPs enter the cells together with cargo molecules[34] such that the cargo molecules tend to be trapped in endosomes. The TatU1A/RNA complexes were indeed mostly localized to endosomes after cell entry, and did not induce RNAi, which occurs in the cytoplasm.[31b] Therefore, we devised the photosensitizing carrier (CPP-RBP-dye) mentioned above, for example, the TatU1A-dye, for the light-dependent release of the TatU1A/RNA complex from the endosome (Figure 4). As mentioned earlier, several groups have used photosensitizers to generate reactive oxygen species, presumably singlet oxygen ($^1O_2$), in a light-dependent manner, to disrupt the endocytic vesicle and to release the target molecules to the cytosol.[22b] In our experiments, a fluorescent dye was used as a photosensitizer, and we found that TatU1A attached to Alexa Fluor 546 (TatU1A-Alexa546) generated $^1O_2$ light-dependently (our unpublished result). TatU1A-Alexa546 bound specifically to a *U1A*-fused cargo RNA, delivered the cargo RNA to cells via the endocytotic pathway, and allowed dispersion of the RNA throughout the cytosol following photostimulation.[31b] Furthermore, light-dependent RNAi-mediated gene silencing was demonstrated.[31b] The RNAi efficiency with the TatU1A-Alexa546 carrier was examined by evaluating the knockdown of EGFP expression in CHO cells stably expressing EGFP. A dramatic light-dependent reduction in EGFP expression was observed in cells treated with a TatU1A-Alexa546/shRNA(anti-EGFP) complex. The treatment of cells with the TatU1A-Alexa546/shRNA complex followed by photostimulation (530−550 nm, 10 J/cm^2) induced a 70% decrease in EGFP expression when compared with the control cells treated with a buffer without the carrier/RNA complex. In contrast, no decrease in EGFP expression was observed when the cells were treated with the TatU1A-Alexa546/shRNA complex and not irradiated. Remarkably, the TatU1A-Alexa546/shRNA complexes were completely trapped in endosomes, and no "leakage" activity was observed with this method.

More recently, several new carrier proteins (TatU1A variants and CPP-RBPs) have been designed for use with the CLIP-RNAi strategy. Most of these worked in a similar manner to TatU1A, and one of the TatU1A variants improved the light-dependent RNAi efficiency when compared to TatU1A.[35] TatU1A with a C-terminal extension of the degradation signal peptide was designed based on the assumption that the degradation of carrier proteins in the cytoplasm promotes the dissociation of the carrier protein from the shRNA and accelerates RNAi. As expected, the C-terminally signal peptide-tagged TatU1A improved the RNAi efficiency by up to ~80%, despite the slight difference with TatU1A-Alexa546.

This photoinduced RNAi method can be used at visible light wavelengths ranging from 530 to 640 nm,[31c] which are less toxic than UV light. We showed that the cells treated with the TatU1A-dye/RNA complexes were not damaged by visible light, and the cytotoxicity associated with the CLIP-RNAi method was considerably lower than that observed with RNA transfection using commercial reagents.[31b] Therefore, the CLIP-RNAi strategy is useful not only for functional analysis but also in therapeutic approaches such as RNAi-mediated photodynamic therapy.

Photothermal Transfection

Gold nanostructures have drawn increasing attention due to their unique optical properties such as strong surface plasmon resonance (SPR) absorption at visible and NIR wavelengths. It has been reported that gold nanoconjugates exhibit acceptable levels of toxicity in vivo and in vitro,[36] although the

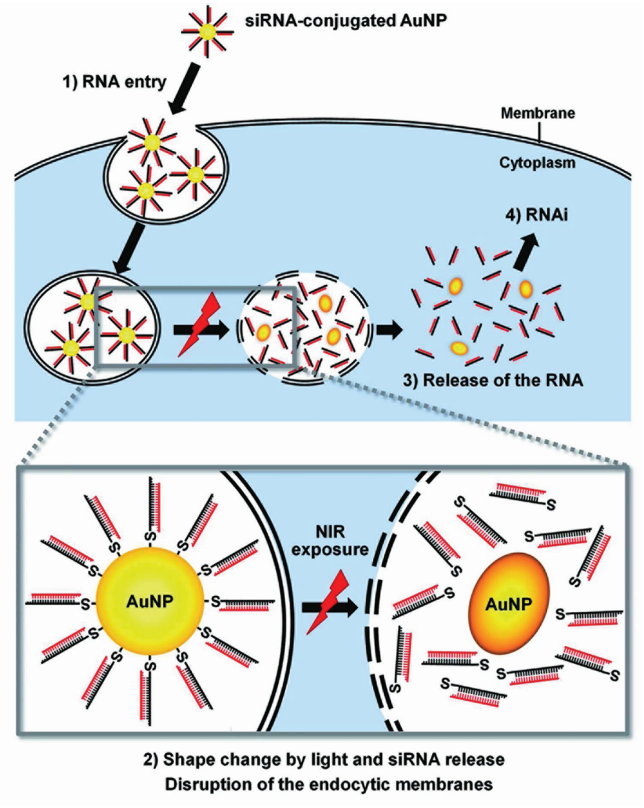

FIGURE 5. Scheme of photothermal transfection for photoinduced RNAi. The siRNA-conjugated AuNPs are internalized by cells via an endocytotic pathway similar to the PCI and CLIP-RNAi methods. Light-dependent release of thiolated siRNAs from AuNPs occurs via the melting of AuNPs accompanied by a shape transformation to spheres. At the same time, disruption of the endocytic membranes is also elicited. See details in text.

toxicity is dependent on the size of the conjugate.[37] Recently, gold nanostructures have been reviewed in detail[38] and are thus only briefly described here. Photothermal therapy is a method of clinical treatment whereby diseased cells and tissues are destroyed by a combination of light and gold, and gold nanorods and nanoshells are being developed as photothermal therapy agents.[39] Unlike PDT, in photothermal therapy, light absorption leads to heating, which causes cell death. Photoirradiation of cells containing gold nanostructures induces SPR at the gold-air interface, and evokes an increase in the temperature of the cells leading to cell death. In addition, Wijaya et al. demonstrated in vitro (without using cells) that thiol-conjugated DNA oligonucleotides could be released from gold nanorods using light-dependent melting of nanorods accompanied by a shape transformation to spheres.[40]

Recently, temporally and spatially controlled RNAi has been demonstrated using gold nanoparticles (AuNPs) conjugated to siRNA (Figure 5).[41] The first application of AuNPs to RNAi was described by Giljohann et al. in 2009.[42] The siRNA-AuNPs synthesized for siRNA delivery were able to enter cells without the use of transfection agents and exhibited an enhanced resistance to RNases compared to naked siRNA. The silencing effect on a target gene was improved simply by incubating cells with siRNA-AuNPs as compared to transfecting cells with naked siRNA using Lipofectamine 2000. However, to date, photoinduced RNAi using AuNPs has not been demonstrated by this group.

In a recent paper, Braun et al. described a new approach using a combination of laser-dependent desorption of thiolated siRNAs from AuNPs and siRNA/AuNP release from endosomes.[41a] The AuNPs employed had a unique combination of features: small size (a diameter of ~40 nm and ~3 nm thick shells), a hollow interior, and a tunable absorption band (near 800 nm). The surfaces of the AuNPs were modified with a polyethylene-glycol-thiolated siRNA duplex against EGFP, and were then coated with a Tat peptide-lipid cell-internalizing agent (Tat-lipid) to allow the use of low AuNP concentrations for stimulation of the macropinocytosis endocytic pathway.[43] The most noticeable difference between the laser-activated gene silencing and cell death induction was the irradiation dose; the energy of the pulsed NIR laser used for the laser-activated gene silencing was significantly lower than that for photothermal therapy.[44,41a] In a previous study by the same group, heating of hollow gold nanoshells with ~2.3 J/cm^2 per laser pulse caused rapid liposome rupture.[45] Braun et al. consistently showed temporally and spatially controlled gene silencing in vitro through the AuNP-activated release of siRNAs using a pulsed NIR laser (800 nm, ~60 J/cm^2, ~80% RNAi efficiency).[41a] Interestingly, siRNA release from the AuNP surface can occur at a lower power (10 J/cm^2) than the siRNA release from endosomes into the cytosol (50 J/cm^2). Taken together, the combination of surface release of the siRNA and endosomal siRNA release enabled spatiotemporally controlled gene silencing.

Lu et al. reported silencing of the nuclear factor-κB (NF-κB) p65 gene in vitro and in vivo via NIR laser-induced release of the target siRNA from hollow gold nanospheres (HAuNS) into the cytosol, a process known as "photothermal transfection".[41b] The authors introduced folic acid to the gold surface of HAuNS particles through a thioctic acid-terminated PEG linker together with a thiolated siRNA against NF-κB p65 (F-PEG-HAuNS-siRNA). The F-PEG-HAuNS-siRNA designed for this experiment specifically targeted folate receptor-positive cells through the folic acid, and was internalized by cells via an endocytotic pathway. Following irradiation (800 nm, 3 J/cm^2), the F-PEG-HAuNS-siRNAs were

released into the cytosol from the endosomes in which they had been trapped. Moreover, the treatment of cells with the F-PEG-HAuNS-siRNA followed by photostimulation induced a 92% decrease in NF-κB p65 expression when compared with the control cells treated with F-PEG-HAuNS-siRNA$_{luc}$ (designed as a control siRNA against luciferase mRNA). Lu et al. reported that the light-dependent endosomal escape of the nanoparticles occurred coincidentally with siRNA desorption from the HAuNS particles. siRNA desorption was purported to result from a shape transformation of the particles, although no direct evidence for this was presented. The photothermal transfection was also tested in a mouse model. As observed in vitro, the incorporation efficiency of the F-PEG-HAuNS-siRNA was higher than that of a PEG-HAuNS-siRNA, although nontargeted tissues such as liver and spleen incorporated a large amount of HAuNS. Furthermore, the authors showed that combined treatment with a DNA synthesis inhibitor (irinotecan) and the F-PEG-HAuNS-siRNA enhanced the apoptotic response to folate receptor-positive cells compared to treatment with irinotecan alone, and consequently, tumor enlargement was delayed.

Thus, the application of AuNPs to spatiotemporally controlled RNAi is an innovative strategy, although there is still room for improvement. The successful implementation of photothermal transfection for future clinical applications without side effects will require stringent regulation of laser-dependent endosomal siRNA release and desorption of thiolated siRNAs from AuNPs.

Conclusion and Perspectives

This Account highlights photoinducible RNAi methods that use a wide variety of technologies, such as caged compounds, photodynamic action, and the photothermal effects of AuNPs. These technologies have long been investigated for use in a wide range of biological processes in vitro and in vivo, and for clinical applications.[3,4,22,38a] In contrast, photoinducible RNAi methods using these technologies have only recently been reported.

The photoinduced RNAi strategies described here were achieved through diverse mechanisms, although all the methods were triggered by light. The caged siRNA strategy utilizes a photochemical deprotection mechanism for RNAi in which the photochemical removal of caging groups from caged siRNAs results in RNAi (Figure 1B). For the PCI and CLIP-RNAi strategies, light-activation of the photosensitizers induces disruption of the endosomal membranes, probably due to ROS generation, and allows endosomal escape of the siRNAs (Figures 3A, 4). The fundamental distinction between PCI and CLIP-RNAi is how the photosensitizers are used. In the PCI method, photosensitizers are introduced into cells separately from the siRNAs and are localized mainly to endocytic membranes, while in the CLIP-RNAi method, a photosensitizer is covalently attached to the RNA carriers and is thus introduced into the endocytic vesicle concomitantly with the siRNA. In the case of photothermal transfection, the conversion of light energy into thermal energy induces a shape transformation of AuNPs, which leads to the release of thiolated siRNAs (Figure 5). Thus, even though all the methods described here are categorized as photoinduced RNAi technologies, the processes for gene silencing are distinctly different.

Most importantly, further optimization of these methods is needed for their expanded use in biological, biotechnological, and therapeutic applications. To this end, it is necessary to regulate the light-dependent induction of RNAi in a stringent manner. In addition, the safety and reactogenicity of the most of the compounds used in these methods should be assessed further for in vivo applications. Success of the spatiotemporal control of gene silencing will facilitate the discovery of novel insights into gene functions and clinical applications without side effects.

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FOOTNOTES

The authors declare no competing financial interest.

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