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ARTICLE *in* BIOCONJUGATE CHEMISTRY · SEPTEMBER 2011

Impact Factor: 4.51 · DOI: 10.1021/bc200411n · Source: PubMed

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Published in final edited form as:
Bioconjug Chem. 2011 October 19; 22(10): 2136–2142. doi:10.1021/bc200411n.

Stabilization and Photochemical Regulation of Antisense Agents through PEGylation

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Abstract

Oligonucleotides are effective tools for the regulation of gene expression in cell culture and model organisms, most importantly through antisense mechanisms. Due to the inherent instability of DNA antisense agents, various modifications have been introduced to increase the efficacy of oligonucleotides, including phosphorothioate DNA, locked nucleic acids, peptide nucleic acids, and others. Here, we present antisense agent stabilization through conjugation of a polyethylene glycol (PEG) group to a DNA oligonucleotide. By employing a photocleavable linker between the PEG group and the antisense agent we were able to achieve light-induced deactivation of antisense activity. The bioconjugated PEG group provides stability to the DNA antisense agent without affecting its native function of silencing gene expression via RNase H-catalyzed messenger RNA degradation. Once irradiated with UV light of 365 nm, the PEG group is cleaved from the antisense agent leaving the DNA unprotected and open for degradation by endogenous nucleases, thereby restoring gene expression. By using a photocleavable PEG group (PhotoPEG), antisense activity can be regulated with high spatial and temporal resolution, paving the way for precise regulation of gene expression in biological systems.

Introduction

Antisense oligonucleotides are extensively used in cell and developmental biology to inhibit gene expression. Site-specific hybridization of antisense agents to target mRNAs suppresses translation, either by degradation of the mRNA though an RNase H-dependent mechanism or by steric blocking of the ribosome.[1-3] Intracellularly, non-modified deoxyribonucleic acids are quickly degraded, mainly by 3′ exonucleases,[4] limiting the applicability of unmodified DNA as antisense agents. For the past two decades, modifications have been made to oligonucleotides to improve the pharmacokinetics and physicochemical properties. Various modifications to the phosphate backbone and the ribose have been developed to improve the stability and half-life of oligomers, including 3′-3′ inverted thymidines,[5] phosphorothioate linkages,[6] locked nucleic acids (LNA),[7] and 2′-*O*-methyl groups.[8]

PEGylation, the covalent attachment of a polyethylene glycol polymer, enhances the pharmacokinetic properties of both oligonucleotides[9] and proteins[10-12] by shielding them from nucleases and proteases thus increasing their stability. Studies have also shown that PEGylation enhances the absorption, bioavailability, and biodistribution of biological macromolecules while reducing toxicity and immunological effects.[9] In addition, PEGylated

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Supporting Information Available: PEGylated antisense agent stability assays and melt curves of DNA:RNA hybridization. This information is available free of charge via the Internet at http://pubs.acs.org.

triplex forming oligonucleotides and siRNAs have shown to be more efficiently taken up by cells, and have displayed improved stability.[13, 14]

We hypothesized that the combination of oligonucleotide PEGylation in conjunction with a light-cleavable linker group would enable photochemical control over antisense agent stability and activity. Light represents an ideal external control element for the study of gene expression and protein function with high spatial and temporal resolution.[15-18] Light-removable protecting groups ("caging groups") have been applied to the light-regulation of DNA function through the inhibition of the oligonucleotide's ability to undergo Watson-Crick base-pairing until the groups are removed through light irradiation.[19-31] Here, we propose that a Photo-PEGylated antisense agent will inhibit gene expression (Scheme 1A) until irradiation with non-damaging UV light removes the PEG group, leading to intracellular degradation of the oligonucleotide and subsequent activation of gene expression (Scheme 1B). Advantages of this approach compared to existing methods of photochemical regulation of antisense function are that a) it can be easily applied to standard, commercially available DNA, b) it stabilizes the DNA under physiological conditions, and c) it enables the light-deactivation of antisense function and thus activation of gene expression.

Experimental Procedures

Synthesis and Purification of PEGylated Oligonucleotides

A 3′ amino-modified phosphodiester DNA oligonucleotide (10 μM, Integrated DNA Technologies) was 5′ radiolabeled with ^{32}P-λ ATP (MP Biomedicals) using T4 polynucleotide kinase (New England Biolabs). The 5′ ^{32}P–DNA-NH_2 (2 μM) was reacted with *N*-hydrosuccinimide (NHS) ester PEG (200 μM, Laysan Bio, Inc) in phosphate saline buffer (PBS, pH 8.0) and incubated for 4 h at 30 °C. Due to incomplete PEGylation, the PEGylated DNA was gel purified, electro-eluted, and concentrated using a Millipore centricon (3000 Da molecular weight cut off). The PEGylated DNA was quantified using a standard curve on a 20% denaturing PAGE gel, through scanning on a Typhoon FLA 7000 and measuring the band intensities using ImageQuantTL.

Melting Temperature Measurements

The melting temperature (T_m) of each PEGylated DNA:RNA duplex was measured using a Cary 100 Bio UV/Vis spectrometer with a temperature controller (Varian). The PEGylated DNA antisense agent and RNA substrate (0.5 μM) were incubated in buffer (0.15 M NaCl, 0.05 M NaH_2PO_4, pH 7.2). The samples were heated to 100 °C for 2 min, and then cooled to 20 °C at a rate of 2 °C/min, held at 20 °C for 5 min, and then heated to 100 °C at a rate of 2 °C/min. Absorbance was recorded at 260 nm every 1 °C. The T_m was determined by the maximum of the first derivative of the absorbance vs. temperature plot. Standard deviations were calculated from three independent experiments.

RNase H Assay

The RNase H assay was performed as described by Tang *et al*.[32] with the following modifications: PEGylated DNA (10 nM) and RNase H buffer were equilibrated, followed by the addition of radiolabeled RNA (^{32}P-γ ATP, 1 μM). The mixture was incubated at 37 °C for 20 min, and RNase H (New England Biolabs, 1 U) was added, followed by an additional incubation for 1 h at 37 °C. Samples were analyzed on a 10% denaturing PAGE gel and scanned on a Typhoon FLA 7000.

Photochemical Regulation of Antisense Activity in Mammalian Cells

HEK 293T cells were passaged into a poly-L-Lys coated 96-well plate, grown to 70% confluency, and transfected with pEGFP-N1 (Clontech, 150 ng), pDsRed-monomer

(Clontech, 150 ng), and antisense agents (25 pmol) using X-tremeGENE (Roche). pEGFP-N1 encodes enhanced green fluorescent protein (EGFP) and served as the transfection control. pDsRed-monomer encodes a red fluorescent protein (DsRed) and was the targeted gene in this study. After 4 h, media was removed, the cells were irradiated on a transilluminator (365 nm, 25 W), and DMEM media was added. Following a 48 h incubation at 37 °C, 5 % CO_2, the media was removed and the cells were imaged on a Jenco inverted microscope. The cells were lysed and the fluorescence was measured on a plate reader (EGFP 485/507 ex/em, DsRed 557/585 BioTek Synergy 4 Microplate Reader).

Spatial Control of Gene Expression in Mammalian Cells

NIH 3T3 cells were passaged into a black 96-well plate and grown to 75% confluency, followed by transfection with pEGFP-N1 (150 ng), pDsRed-monomer (150 ng), and antisense agent (25 pmol) using X-tremeGENE. After 4 h, the media was removed and half of the well was covered with an aluminum foil mask. The cells were irradiated for 2 min on a transilluminator (365 nm, 25 W) and DMEM media was added. Following a 48 h incubation, the media was removed and the cells were imaged on a Zeiss Axio Observer inverted microscope (5x magnification, filter sets 43 HE DsRed and 38 HE eGFP).

Results and Discussion

First, the PEGylated DNA was synthesized through the acylation of 3′ amino-modified oligonucleotides with *N*-hydroxysuccinimide-activated polyethylene glycols of 5 kDa, 20 kDa or 40 kDa (Scheme 2A). A DNA sequence targeting the DsRed gene was selected for subsequent cell-based reporter assays[27, 33] The PEGylated antisense agents were purified through gel electro-elution (Scheme 2C). In order to validate that the PEG group will stabilize the non-modified DNA against nucleases, the 3′ PEGylated oligonucleotides were incubated in DMEM growth media (10% FBS). Upon conjugation of the 5 kDa PEG group to the deoxyoligonucleotide, the stability increased from 4 hrs to 24 hrs. The 20 kDa PEG and the 40 kDa PEGylated oligonucleotides displayed similar trends with stabilizing DNA for a minimum of 24 hrs. The stability of the PEGylated oligonucleotides was also tested in the presence of exonuclease I, by measuring oligonucleotide integrity over time. A 15-, 30-, and 60-fold increased stability was found for DNA PEGylated with 5, 20, and 40 kDa PEG reagents, respectively. Thus, increasing the size of the bioconjugated PEG from 5 kDa to 40 kDa, increases the stability of the oligonucleotide in both DMEM media and in the presence of an exonuclease (Supporting Information, Figure S1 and S2).

Melt curves of the PEGylated antisense agents were measured in the presence of target mRNA (5′ GCAGGAGGCCUCCUCCGAGAACGUCAUCACCGAGUUCAUCAAGGU 3′) to ensure that the PEG groups do not affect DNA:RNA hybridization. The non-PEGylated antisense agent had a T_m of 69 °C (Table 1). The conjugation of the 5 kDa PEG to the DNA phosphodiester oligonucleotide did not interfer with DNA:RNA hybridization as the T_m slightly decreased by 6 °C when compared to the non-PEGylated DNA. With an increasing PEG size to 20 kDa and 40 kDa, the T_m decreased by 13 °C and 19 °C, respectively, but hybridization was still detected (see Supporting Information, Figure S3). Therefore, all PEGylated deoxyoligonucleotides undergo DNA:RNA hybridization and thus have potential to function as antisense agents in live cells. The attachment of a smaller PEG group (5 kDa) had a less pronounced effect on melting point depression than larger PEG groups (20 kDa or 40 kDa).

Since the PEG groups do not affect DNA:RNA hybridization, the activity of the PEGylated DsRed antisense agents was determined in cell culture. Human embryonic kidney (HEK) 293T cells were co-transfected with pEGFP-N1, pDsRed-monomer and the PEGylated antisense agents. The PEGylated antisense agents target the DsRed mRNA while the

enhanced green fluorescent protein (EGFP) served as a transfection control; therefore, DsRed fluorescence was normalized to EGFP fluorescence. The non-modified antisense agent (AA) showed no knockdown of DsRed expression (Figure 1A), presumably due to a rapid intracellular degradation of the deoxyoligonucleotide (as discussed above). As a positive control, a hairpin loop-protected DsRed antisense agent that has previously been shown to successfully inhibit DsRed expression was used.[27, 33] This hairpin antisense agent (AA-HP) produced a 74% inhibition of DsRed expression (Figure 1B). Gratifyingly, all PEGylated antisense agents (AA-5K, -20K, and -40K) achieved similar levels of DsRed suppression (58-68%). Since the knockdown is not statistically different ($p \geq 0.15$), it can be concluded that even small PEG groups of 5-20K are sufficient for the protection of small deoxyoligonucleotides. Thus, the ability of non-modified, PEGylated DNA to act as a translational-silencing antisense agent in tissue culture was demonstrated for the first time.

Antisense agents can inhibit gene function via two pathways: a) the antisense agent can sterically block the translation of the mRNA or b) the mRNA:DNA duplex can recruit RNase H to bind to the duplex and degrade the mRNA.[2] In order to determine by which mechanism the PEGylated antisense agent inhibits gene expression, an RNase H assay was performed. The DsRed mRNA target in the absence of antisense DNA was not affected by the addition of RNase H (Figure 2, Lanes 1-2). Non-modified DsRed AA was incubated with its complementary mRNA sequence in the presence and absence of RNase H. As expected, only upon addition of RNase H was the mRNA degraded (Figure 2, Lanes 3-4). The same result was obtained for the PEGylated AA-5K DNA and RNase H (Figure 2, Lanes 5-6), indicating that the presence of the PEG group has no effect on the recruitment of RNase H. Thus, the PEGylated antisense oligonucleotide most likely inhibits gene expression intracellularly through RNase H-mediated degradation of mRNA.

Based on the results presented above, PEGylated oligonucleotides are active and stable antisense agents in cell culture, while the non-PEGylated DNA is quickly degraded. Thus, we hypothesized that we can photochemically inactivate an antisense agent by inserting a light-cleavable linker between the PEG group and the DNA (Scheme 1C). In order to test this hypothesis, we used a previously synthesized 5 kDa PhotoPEG reagent[34] and additionally synthesized a 20 kDa PhotoPEG group. The DsRed antisense DNA was PEGylated with the PhotoPEG group as shown in Scheme 2B.

The photoactivation of gene expression was conducted by transfection of the 5 kDa and 20 kDa PhotoPEGylated antisense agents with pEGFP-N1 and pDsRed-monomer into HEK 293T cells. The cells were irradiated for 2 min using a transilluminator (365 nm, 25 W), followed by incubation for 48 hrs. As in the previous cellular experiments, EGFP was used as a transfection control and DsRed fluorescence was normalized to EGFP fluorescence. The 5 kDa PhotoPEG antisense agent (AA-5K PhotoPEG) reduced DsRed expression by 52% (similar to earlier results shown in Figure 1), and after irradiation DsRed expression was fully restored – comparable to the no antisense agent control (Figure 3A and B). The 20 kDa PhotoPEG antisense agent (AA-20K PhotoPEG) almost completely inhibited DsRed expression (80% inhibition), while after irradiation, fully restored DsRed expression was observed as well. Gene silencing with the AA-20K PhotoPEG reagent was more pronounced than what was previously observed for the simple PEG reagent without the light-cleavable linker (Figure 1). However, based on our cellular stability assays (see Supporting Information), the AA-20K PEG was more stable than the AA-5K PEG toward enzymatic degradation. This demonstrates the excellent switching from virtually complete inhibition of gene expression before irradiation to fully restored gene expression after irradiation. For the first time, non-modified, standard DNA was conjugated with a photo-cleavable PEG group and used in the photochemical activation of gene expression.

Having successfully achieved light-activation of gene expression with an excellent off/on ratio by using the PhotoPEGylated antisense agent, we next investigated the possibility of spatial control over DsRed expression in mammalian cells. In a 96-well plate, the AA-20K PhotoPEG was co-transfected into NIH 3T3 cells with pEGFP-N1 and pDsRed-monomer. Only one half of a well was then irradiated for 2 min (365 nm, 25 W), followed by incubation for 48 hrs. Consistent with earlier observations (Figure 4), the non-modified antisense agent (AA) exhibited no inhibition of DsRed expression (Figure 4), conversely the hairpin antisense agent (AA-HP) completely inhibited DsRed expression. Cells transfected with the 20 kDa PhotoPEG antisense agent were only irradiated on the left half of the well and DsRed expression is only observed within the irradiated area. In the non-irradiated area, the right side of the micrograph, no DsRed expression is visible due to an intact PEGylated DsRed antisense agent. Thus, a high level of spatial control over gene activation was achieved.

In summary, PhotoPEG antisense agents were developed that enable precise activation of gene expression through UV irradiation. Traditionally, antisense agents are stabilized by various backbone and sugar modifications for applications in tissue culture and whole organisms. These modifications are often synthetically challenging and, moreover, prevent RNase H-catalyzed mRNA degradation. If RNase H cleavage of mRNA is desired, mixed modified/non-modified deoxyoligonucleotides need to be synthesized. We discovered that simple PEGylation stabilizes non-modified DNA intracellularly with minimal to no effects on antisense activity, DNA:RNA hybridization, and RNase H-catalyzed mRNA degradation, thus providing efficient and readily synthesized antisense agents. The application of a light-cleavable PhotoPEG linker inserted between the PEG polymer and the oligonucleotide enabled sequence-specific gene silencing until cells were briefly irradiated with UV light of 365 nm, inducing PEG cleavage and subsequent rapid, intracellular degradation of the antisense oligonucleotide. Degradation of the antisense agent then in turn activates the expression of the previously silenced gene of interest. Moreover, in addition to temporal control over gene expression, the developed methodology introduced a simple means for spatial control over gene activation through locally restricted irradiation of a monolayer of mammalian cells. This approach (light-*activation* of gene expression) is complementary to classical antisense caging methodologies (light-*deactivation* of gene expression). Due to the straightforward design of the reagents, only requiring standard deoxyoligonucleotides, the developed methodology can be readily applied in any chemical biology, cell biology, and molecular biology lab.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported in part by the National Institutes of Health (R01GM079114), the Beckman Foundation (Beckman Young Investigator Award for AD), and the Research Corporation (Cottrell Scholar Award for AD).

Abbreviations

PEG	polyethylene glycol
PhotoPEG	photocleavable polyethylene glycol
NHS	*N*-hydroxysuccinimide
PBS	phosphate saline buffer

HEK	human embryonic kidney cells
AA	antisense agents
AA-HP	hairpin-protected antisense agent
AA-5K	PEGylated 5 kDa antisense agent
AA-20K	PEGylated 20 kDa antisense agent
AA-40K	PEGylated 40 kDa antisense agent
AA-5K PhotoPEG	5 kDa photocleavable PEGylated antisense agent
AA-20K PhotoPEG	20 kDa photocleavable PEGylated antisense agent

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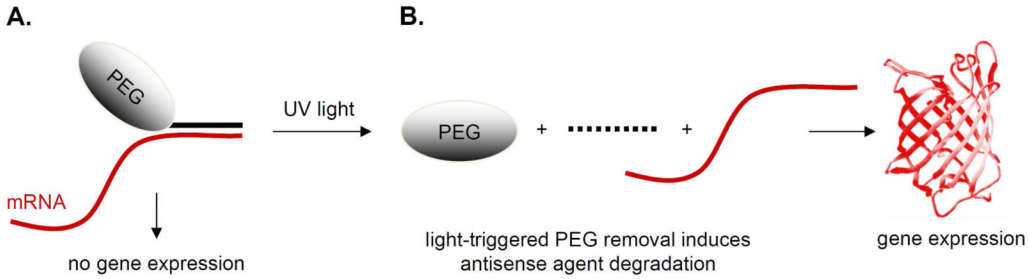
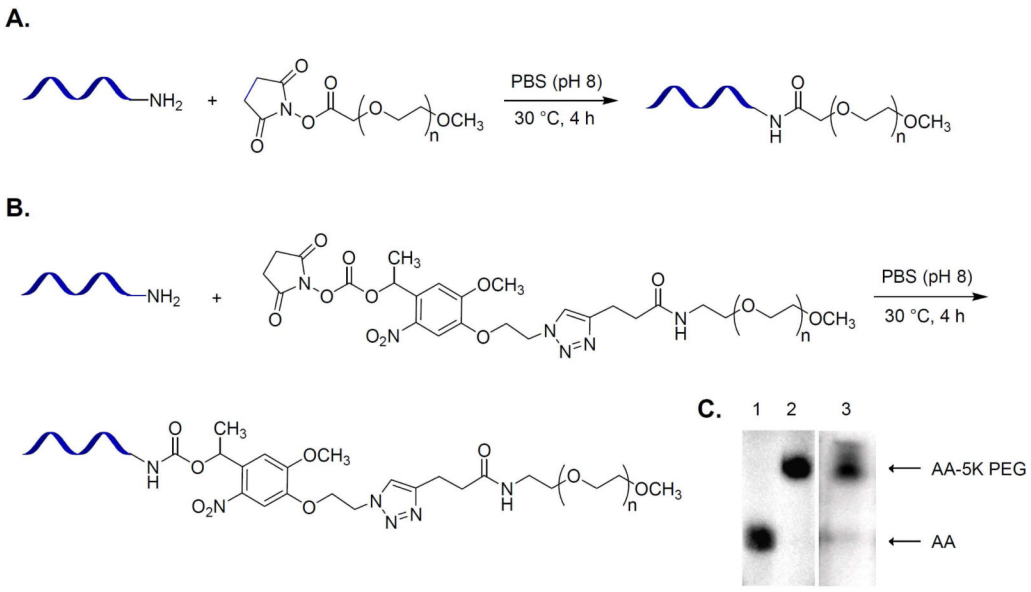


Scheme 1.

Photoregulation of antisense activity with light-removable PEG groups. **A**. A PEGylated antisense agent binds to the mRNA target sequence, which recruits RNase H to the mRNA:DNA duplex leading to the degradation of the mRNA and thus gene silencing. **B**. A PhotoPEG-antisense is cleaved upon UV irradiation, leaving the DNA-based antisense agent susceptible to degradation by exonucleases, thereby allowing the translation of the mRNA and gene expression.



Scheme 2.

PEGylation of 3′ amino-modified DNA A. PEGylation reaction of 3′ amino-modified DNA with N-hydroxysuccinimide PEG. B. PEGylation and decaging of DsRed targeting antisense DNA with the PhotoPEG reagent. **C**. Representative PAGE analysis of purified, PEGylated DNA. Lane 1: non-modified antisense agent (AA). Lane 2: 5 kDa PEGylated antisense agent (AA-5 kDa PEG). Lane 3: 20 kDa photocleavable PEGylated antisense agent (AA-5K PhotoPEG).

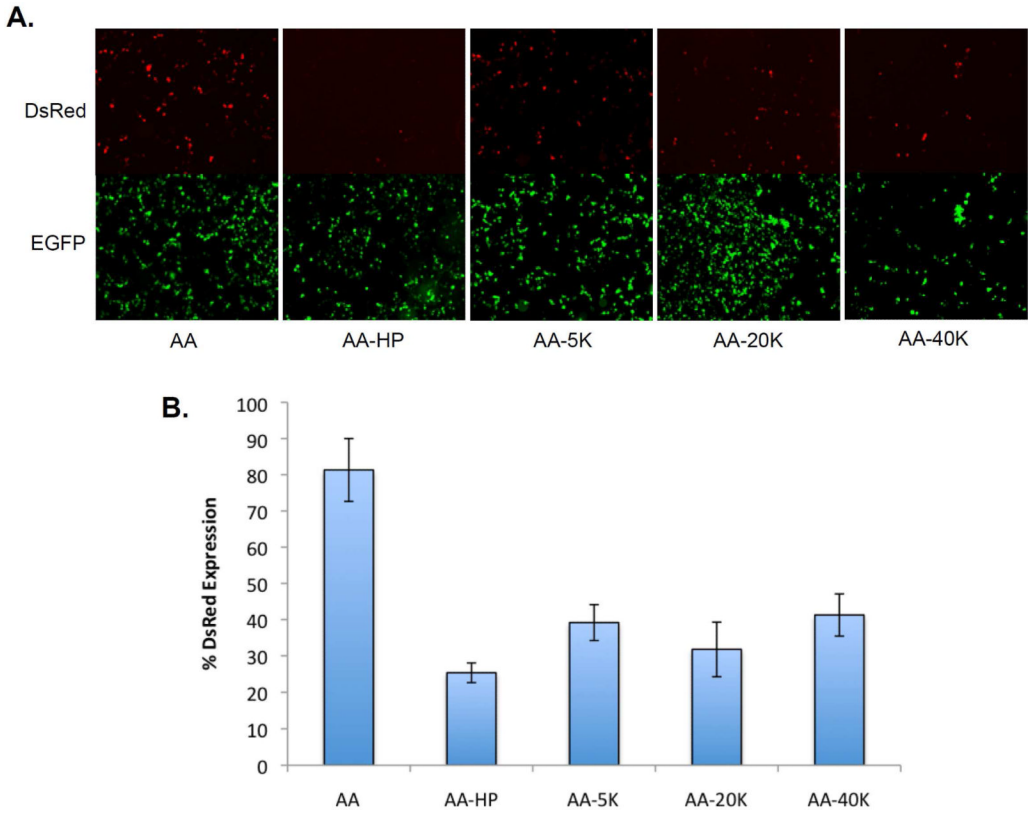


Figure 1.

Inhibition of DsRed expression by PEGylated antisense agents. HEK 293T cells were transfected with pEGFP-N1, pDsRed-Monomer and antisense agent (25 pmol). **A**. After a 48 h incubation, the media was removed and the cells were imaged. The DsRed channel is shown above the EGFP channel. **B**. Cells were subsequently lysed and the fluorescence was measured on a BioTek plate reader. DsRed (557/585 nm) fluorescence was normalized to EGFP (485/507 nm) fluorescence. Error bars represent standard deviatons from three independent experiments. AA: non-modified antisense agent. AA-HP: hairpin antisense agent. AA-5K: 5 kDa PEGylated antisense agent. AA-20 kDa: 20 kDa PEGylated antisense agent. AA-40: 40 kDa PEGylated antisense agent.

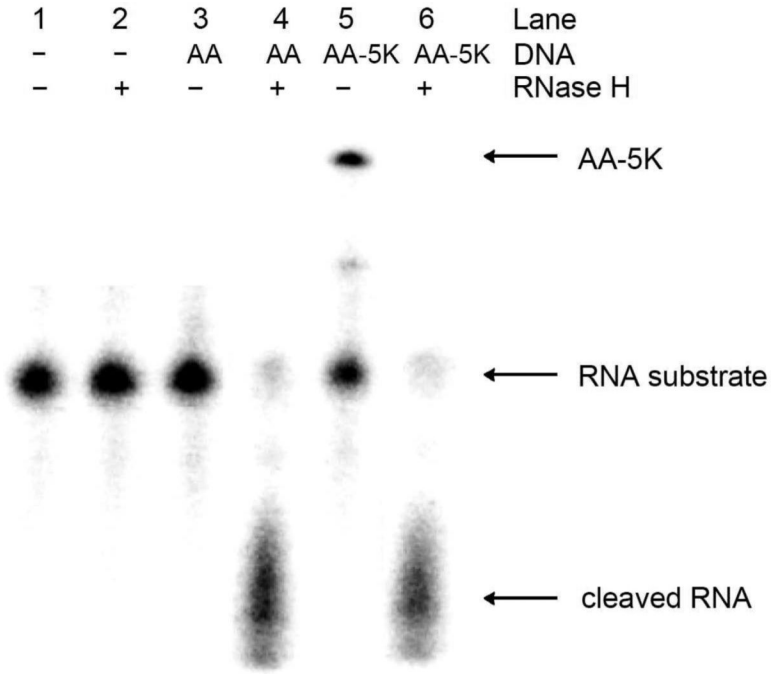


Figure 2.

The PEGylated antisense agent induces RNA degradation through an RNase H mediated mechanism. AA and AA-5K PEG (10 nM) were incubated with a radiolabeled RNA substrate (1 μM) and RNase H (1 U) for 1 h at 37 °C. Samples were analyzed on a 10% denaturing gel and imaged with a Typhoon FLA 7000. AA: non-modified antisense agent. AA-5K: 5 kDa PEGylated antisense agent.

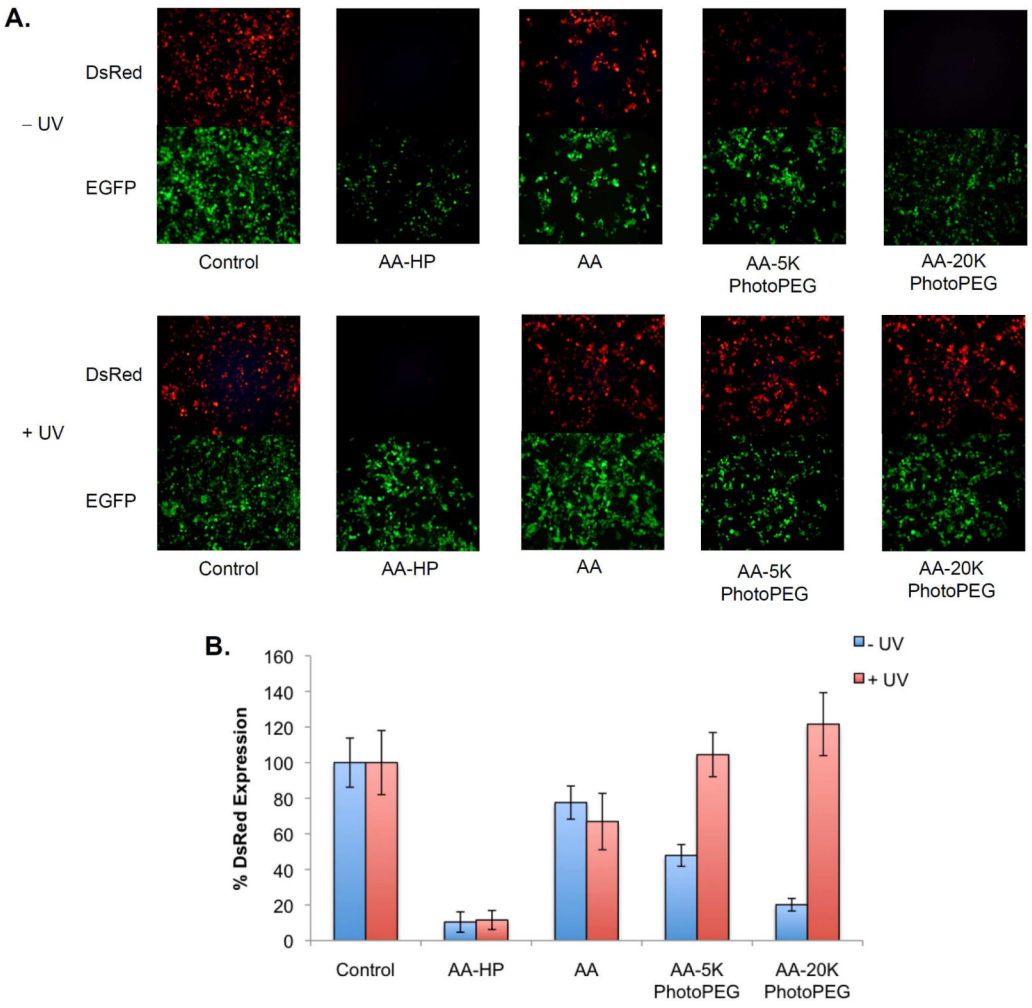


Figure 3.

Light-activation of gene expression using a PhotoPEGylated antisense agent. HEK 293T cells were transfected with pEGFP-N1, pDsRed-Monomer, and antisense agent. Cell were irradiated for 2 min (25 W, 365 nm) or kept in the dark. **A**. The cells were imaged after 48 h. The DsRed channel is shown above the EGFP channel. **B**. The cells were then lysed, and the fluorescence was measured on a BioTek plate reader. DsRed (557/585 nm) fluorescence was normalized to EGFP (485/507 nm) fluorescence. Error bars represent standard deviations from three independent experiments. AA-HP: hairpin antisense agent. AA: non-modified antisense agent. AA-5K PhotoPEG: 5 kDa photocleavable PEGylated antisense agent. AA-20K PhotoPEG: 20 kDa photocleavable PEGylated antisense agent.

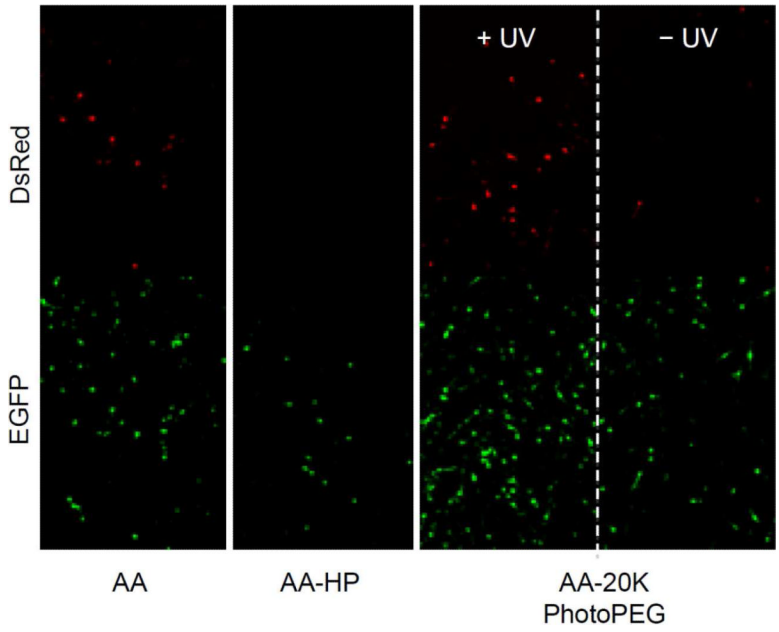
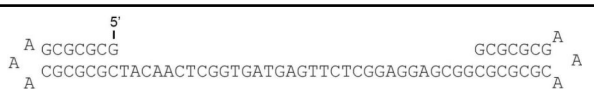


Figure 4.

Spatial activation of gene expression using a PhotoPEGylated antisense agent. NIH 3T3 cells were transfected with pEGFP-N1, pDsRed-Monomer, and AA-20K PhotoPEG. Only one half side of the well was irradiated for 2 min (25 W, 365 nm) and the cells were imaged after 48 h. The DsRed channel is shown above the EGFP channel. AA: non-modified antisense agent. AA-HP: hairpin antisense agent. AA-20K PhotoPEG: 20 kDa photocleavable antisense agent.

Table 1

Sequences and melting temperatures of PEGylated DsRed antisense agents. Standard deviations were derived from T_m measurements in triplicate.

Name	DNA Sequence	T_m / °C
AA	5’ AACTCGGTGATGACGTTCTCGGAGGAG-NH_2	69.2 ± 0.7
AA-HP		61.1 ± 0.8
AA-5K	5’ AACTCGGTGATGACGTTCTCGGAGGAG-NH-PEG_{5kDa}	63.4 ± 1.3
AA-20K	5’ AACTCGGTGATGACGTTCTCGGAGGAG-NH-PEG_{20kDa}	56.1 ± 1.5
AA-40K	5’ AACTCGGTGATGACGTTCTCGGAGGAG-NH-PEG_{40kDa}	50.9 ± 1.9