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Comparison of Thermally Actuated Retro-Diels-Alder Release Groups for Nanoparticle Based Nucleic Acid Delivery

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Abstract

The present study explores alternate pericyclic chemistries for tethering amine-terminal biomolecules onto silver nanoparticles. Employing the versatile tool of the retro-Diels-Alder (rDA) reaction, three thermally-labile cycloadducts are constructed that cleave at variable temperature ranges. While the reaction between furan and maleimide has widely been reported, the current study also evaluates the reverse reaction kinetics between thiophene-maleimide, and pyrrole-maleimide cycloadducts. Density Functional Theorem (DFT) calculations used to model and plan the experiments, predict energy barriers for the thiophene-maleimide reverse reaction to be greatest, and the pyrrole-maleimide barriers the lowest. Based on the computational analyses, it is projected that the cycloreversion rate would occur slowest with the thiophene, followed by furan, and finally pyrrole would yield the promptest release. These thermally-responsive linkers, characterized by Electrospray Ionization Mass Spectrometry, ¹H and ¹³C NMR, are thiol-linked to silver nanoparticles and conjugate single stranded siRNA mimics with 5' fluorescein tag. Second harmonic generation spectroscopy (SHG) and fluorescence spectroscopy are used to measure

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release and rate of release. The SHG decay constants and fluorescence release profiles obtained for the three rDA reactions confirm the trends obtained from the DFT computations.

Graphical abstract

Keywords

retro-Diels-Alder; delivery; Second Harmonic Generation (SHG); nanoparticle; maleimide; pyrrole; thiophene; furan

Introduction

Delivery of short non-coding nucleic acid molecules as potential therapeutics has widely been investigated [1] [2] [3] [4] [5] [6] [7]. These molecules play a crucial role in regulating gene expression in numerous processes from embryonic to immune system development, and have been sought after as potential therapeutic agents relevant to human physiology and pathology. Clinical adoption, however, has been slow as a result of unidentified off-target effects as well as non-specific and inefficient delivery, requiring a high dosage for effective treatment. With the capability to respond to internal or external stimuli, nanoparticle delivery systems hold great promise for efficient spatiotemporal gene control of therapeutic delivery. Targeted delivery and release of therapeutics can be initiated via physical, chemical, or mechanical cues. Nanoparticle systems designed for nucleic acid caging and controlled release include polymer-based media [8], surface-linked or encapsulated nanoparticles [7] [9], liposomal and viral vectors [10] [11]. These systems can be tailored to employ various cues for nucleic acid delivery at targeted sites, including pH change [12], temperature [13] or electromagnetic-mediated stimuli [14]. Most of these methods rely on the linker or substrate chemical nature for stimuli response and drug release, typically through bond breaking and rearrangement.

For efficient temporal manipulation of small molecules and siRNA, click chemistry, most commonly reported between an azide and alkyne, can provide bioconjugation abilities of biomolecules with specific systems additionally providing a switch-like trigger for

molecular release. These reactions are wide in scope, but pertain to reactions proceeding under mild conditions to give high yields and innocuous by-products [15] [16]. Diels-Alder reactions meet this criteria, and involve formation of a cycloadduct product derived from a dienophile and diene in which more stable σ -bonds are formed from [4+2] π -bonds, based on overlapping electron levels between higher and lower occupied and unoccupied molecular orbitals [17] [18] [19]. At higher temperatures the cycloaddition reactions undergo reverse reaction pathways, referred to as the retro-Diels-Alder (rDA) reaction, to reproduce their diene and dienophile counterparts [20] [21] [22]. In Bakhtiari et al. [23], it was demonstrated that the cycloadduct between a furan and maleimide could be synthesized at room temperature over seven days, with the rDA reaction observed to occur at temperatures 60 °C and above. Using gold nanoparticles modified with fluorescein-tagged furan-maleimide linkers and irradiated at their plasmonic wavelength of 532 nm, an increase in solution fluorescence was observed due to localized plasmon-phonon heat generation, triggering the rDA reaction and releasing the markers. Other groups have utilized chiral auxiliary synthesis methods for enantiomer-selectivity of pyrrole-pyrimidine ring structures and microwave-inducible rDA reaction [24] [25] [26]. The rDA chemistry is a versatile tool for the delivery of short biological molecules utilizing simple chemical modifications, and provides a stimuli-responsive switch to trigger cargo release based on localized heat induction. By designing multiple linkers with alternate thermal responses, temporal delivery of more than one drug for gene therapy can be achieved.

The Diels-Alder forward reaction can be facilitated via an electron-enriched diene with an electron-poor dienophile, substituted with electron-donating and electron-withdrawing groups, respectively [27]. In the current study, we aim to build on the chemistry investigated previously [23], in which the bond breaking of 7-oxa-bicyclo-[2.2.1]hept-5-ene-2,3-dicarboxylic imide was studied, and explore the bicyclic reactions of alternate dienes. With distinct temperature ranges achieved in which to initiate the rDA reaction, multiplex delivery applications can be realized. In addition to the described furan-maleimide composition, substitution of the furan with both a pyrrole and thiophene-based diene was investigated, utilizing chemical modifications of the cycloadduct with a thiol terminal for nanoparticle conjugation, and a carboxyl terminal group for crosslinking with 3' amine-modified nucleic acids (Figure 1). Density functional theory was used to model the reaction barriers at various temperatures for the three different diene reactions with maleimide to aid in the rational design of the system and experimental conditions. To measure the rDA rates for each of the model compounds, second harmonic generation spectroscopy was utilized for nanoparticle surface measurements; the first time the technique is used, to our knowledge, to analyze the reversion of the DA linker.

Materials & Methods

6-Maleimidohexanoic acid (90%), 2-furanmethanethiol (98%), 2-thienylmethanethiol, pyrrole-2-carboxylic acid (99%), dichloromethane (DCM) (99.8%), methanol (MeOH) (>99.9%), cysteamine (95%), N-hydroxysuccinimide (NHS) (98%), silver nitrate (99%), formaldehyde (36.5–38%), sodium hydroxide (98%, pellets), hydroxypropyl cellulose (HPC) (Mn=80,000, 99%), antifoam A (100%), tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP) (0.5M) and dimethyl sulfoxide-d6 (99.9%) were all received

from Sigma Aldrich (St. Louis, MO). EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) was purchased from Thermo Fisher Scientific (Waltham, MA), and custom 3'-amine/ 5'-FAM modified anti-sense RFP single stranded siRNA (UUGGAGCCGUACUGGAACUUG) were purchased from Sangon Biotech (Shanghai, China). All reagents were used as received. A mounted 405 nm LED light (1500mW) from ThorLabs, Inc. (Newton, NJ) was utilized for photorelease experiments.

Computational methods

DFT computations were performed prior to experimentation. The optimized geometry, normal mode analysis, thermodynamic properties, and reaction barriers were calculated using the NWChem (v6.6) software package [28] at a B3LYP and 6-311G* level of theory [29]. Reduced atom models for the maleimide were used to ensure no negative vibrational modes for reactants and products, and just one large negative vibrational mode for the transition states. Optimizations were done using tighter criteria for the gradient, gradient maximum and root mean square, and Cartesian step maximum and root mean square. Settings for transition state searches were performed using optimized structures of the reactants initially constrained to a distance of 2.4 Å for the reacting carbon atoms. The thermodynamic data and zero point energies were obtained from the normal mode analysis calculations at 25, 40, 60, and 80 °C. All calculations were performed as gas phase, ignoring solvent effects.

Synthesis of nanoparticles

Colloidal silver nanoparticles (SNPs) were prepared as described in [30] which yielded nanoparticles of 75 nm in diameter size in that study. Briefly, at room temperature 35 mL of each 125 mM silver nitrate (AgNO₃) and 61.5 mM formaldehyde (HCHO) were incrementally added 0.5 mL/min into a pre-made solution consisting of 0.5 g NaOH, 0.31 g HPC, 330 mL deionized (DI) water (18.2 MΩ), and 5 µL Antifoam A. For purification, the nanoparticles were filtered via dialysis and freeze-dried under vacuum for 72 h before use. A stock solution of 200 ppm in DI water was later prepared for further chemical modification.

Synthesis of Diels-Alder linkers & siRNA attachment

For the cycloaddition between 6-maleimidoheptanoic acid and 2-furanmethanethiol, the protocol outlined in [23] was followed with minor modifications. Briefly, 4.18 g of the dienophile, the maleimide, was combined with 1 mL of the diene reagent, in a 1:1 (v/v) DCM:MeOH solvent mixture. The reaction was allowed to proceed for 7 days under agitating conditions at room temperature in a sealed container. For the Diels-Alder reactions between the 2-thienylmethanethiol (0.5 mL) and the 6-maleimidoheptanoic acid (2.11 g), and the pyrrole-2-carboxylic acid (0.555 g) and 6-maleimidoheptanoic acid (2.11 g), the reagents were again mixed together in MeOH-only solvent. Both the reactions for the pyrrole and thiophene were carried out in an oil bath at 60 °C for 3 days under controlled ventilation. The bicyclic products between the different dienes and dienophile were purified by High Performance Liquid Chromatography (HPLC) and characterized via ESI MS, ¹H and ¹³C NMR. For SNP attachment, the solutions were simply dried with nitrogen gas to remove excess solvent and concentrate the sample prior to suspension, without HPLC purification. Additionally, in the case of the pyrrole-2-carboxylic acid Diels-Alder reaction, the diene was

first crosslinked with cysteamine using EDC coupling chemistry for SNP modification. Briefly, EDC (0.500 g), NHS (0.800 g) and cysteamine (0.400 g) were added to the pyrrole-2-carboxylic acid (0.555 g) and agitated overnight at room temperature. After centrifugation of the 1 mL-aliquoted SNPs (10,000 rpm, 15 min) and removal of the supernatant, 0.5 mL of the Diels-Alder products were added directly to the pelleted nanoparticles. The NP surface modification step was left to proceed at room temperature for 24 hours, for all the three generated products. The nanoparticles were then washed thrice by centrifuging for 10 min at 10,000 rpm consecutively, in which each step involved removal of the supernatant and resuspension in 1 mL of 70 % (v/v) ethanol. To conjugate the FAM-tagged RFP antisense siRNA mimic, the EDC coupling protocol was again used with 100 μ L of an aqueous EDC/NHS (100 mM) stock solution added to each of the resuspended nanoparticle aliquots followed by 50 μ L of the amine-terminated siRNA (4 μ M). After 24 hours, the particles were again centrifuged, washed, and resuspended in DI water. A control sample in which cysteamine modified SNPs were linked to the 6-maleimido hexanoic acid via EDC coupling was also prepared, similarly to the pyrrole-based reaction described above but without addition of the diene, to test the stability of both the amide and thiol linkages. Conjugation of the linkers and siRNA was tested by chemically reducing the Ag-SR bond using TCEP reagent and measuring FAM intensity of the supernatant.

Water-bath heating

The siRNA-functionalized nanoparticles were placed in a water bath and heated at 3 different temperatures, 40, 60, and 80 $^{\circ}$ C, each for duration of 2 h. Release of the siRNA at each temperature was quantified by measuring FAM fluorescence intensity of the supernatant at 485/520 nm (ex/em) (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA), and normalizing to TCEP-treated samples. All fluorescence measurements were also measured against a non-treated sample consisting of modified nanoparticles, to take into account any freely dispersed siRNA. The supernatant of the non-treated sample was established as the baseline and subtracted from the values plotted for all samples.

Second Harmonic Generation measurements

The experimental setup for SHG measurement has been described previously [31]. Briefly, the experimental setup consisted of a Ti: sapphire oscillator laser having 800 nm central wavelength, 80 MHz repetition rate and 70 fs pulse duration with an average power of 2.5 W. For SHG measurements, the 800 nm probe laser at an attenuated, average power of 300 mW was focused into a 1 cm $\times$ 1 cm quartz cuvette containing the sample. The SHG signal from the siRNA-SNP complex was collected in the forward direction in real-time at different sample temperatures using heating tape. A LabVIEW program was used to control a magnetic stir bar, beam block to open and shut every 15 s and to collect background-subtracted time-dependent SHG spectroscopic measurements.

Light activated photothermal release

SNP samples surface-modified with each of the three Diels-Alder and FAM-siRNA constructs were investigated for release via localized heating, by light irradiation at 405 nm using a mounted LED light source (measured output power of 1 W). Solutions of the

modified SNPs were contained in a 35 mm Petri dish and irradiated from the bottom of the dish for 20, 40, 60, and 80 min time intervals. The suspension samples were then collected, centrifuged, washed and treated with TCEP to measure the remaining oligonucleotide on the surface of the nanoparticles, by measuring directly the FAM fluorescence intensity at 485 nm/525 nm (ex/em) in the supernatant. Bulk solution temperature during irradiation was measured with a T-type thermocouple.

High Performance Liquid Chromatography (HPLC)

Diluted samples of the three different products were injected (10 uL) through a Dursan®-coated stainless steel column packed at high pressure with a silica-C18 bed using a binary gradient Shimadzu UHPLC system. The mobile phase consisted of dual flow of acetonitrile and water, with the different compositions used for each DA product listed in the SI document.

Electron-Spray Ionization Mass Spectrometry

Mass spectrometric analysis was performed on a Waters Q-TOF Premier quadrupole/time-of-flight (TOF) mass spectrometer (Waters Corporation (Micromass Ltd.), Manchester, UK). Operation of the mass spectrometer was performed using MassLynx™ software Version 4.1 (<http://www.waters.com>). Samples were introduced into the mass spectrometer using a Waters 2695 high performance liquid chromatograph. The samples were analyzed using flow injection analysis (FIA), in which the sample was injected into the mobile phase flow and passes directly into the mass spectrometer, where the analytes are ionized and detected. The mobile phase used was 90 % acetonitrile (LC-MS grade), and 10 % aqueous 10 mM ammonium acetate. The flow rate was 0.15 mL/min. The nitrogen drying gas temperature was set to 300 °C at a flow of 7 L/min. The capillary voltage was 2.8 kV. The mass spectrometer was set to scan from 100-100 m/z in both positive and negative ion modes, using electrospray ionization (ESI).

Results & Discussion

DFT Computations

DFT methods provide essential quantum mechanical computations of forward and reverse barriers and energies of reactions [32]. The structures used in the pericyclic reaction schemes for the B3LYP/6-311G* set are shown in Table 1, and an example input for one of the optimizations is included in the supporting information (Table S1). The Gibbs free energy and enthalpy barriers are summarized in Table 2. For emphasis, in all three cases the same maleimide structure was used to link the dienes, with variation occurring with only the latter group. The data presented in Table 2 portray a consistent pattern; higher energy barriers for G and H_{rxn} for the retro-Diels-Alder reaction with thiophene than either the pyrrole or furan, while the values for the former diene appear as the lowest. This trend holds true at all temperatures with little variation. Based on the computations, it would suggest that the rDA reaction would occur at higher temperatures with the thiophene than the furan diene, while the pyrrole would initiate release at even lower temperatures.

Synthesis of SNPs & Diels-Alder products

Characterization of the synthesized SNPs has previously been reported in Qureshi et al. [30] [34]. The HPC-stabilized nanoparticles' hydrodynamic radius was determined to be $170 \text{ nm} \pm 1.46$ with a zeta potential of $-10.0 \text{ mV} \pm 0.41$. Additionally, the UV-Vis spectra of the SNPs were collected and reported in the supporting information document (Figure S1).

The furan-based Diels-Alder reaction was performed similarly to the previous procedure [23]. The thiophene and pyrrole reactions, however, required elevated temperatures to proceed as the conditions used for the furan did not yield the desired products after the seven day period. The former two dienes are less electronegative than the furan and as a result possess increased aromaticity [35]. Previous reports have highlighted the difficulty of cycloaddition reactions involving thiophene as a result of its lack of reactivity [36]. Increased temperatures and pressures are required to overcome its reaction barrier to produce modest yields of bicyclic products, as was demonstrated in Margetic's work involving [n]polynorbornanes with thiophene in vectorially aligning S-bridged cyclic structures [37].

Silver nanoparticle conjugation of the thermally-labile linkers was achieved via thiol-linkage. In the case of the pyrrole reaction, cross-linking of the molecule with cysteamine was performed for nanoparticle modification. The terminal thiol group on the bicyclic molecules allows for the facile Ag-SR functionalization on nanoparticle surfaces, as has been described elsewhere [38]. An important note to highlight is that all three dienes were functionalized with a terminal thiol group that could potentially react directly with the maleimide. The thiol-maleimide reaction is a subclass of the thiol-Michael Addition click reactions, which are typically either base- or nucleophile-catalyzed [39] [40]. The former is unlikely to occur in the outlined reactions, as the Diels-Alder cycloaddition was carried out at slightly acidic conditions at a measured mixture pH of 5–6. The dienes themselves are unlikely to be strong enough nucleophiles to react with the C=C bond on the maleimide and remove the H from the –SH group to bond with the maleimide. But more importantly, the Diels-Alder cycloadduct was selected for by surface modification with the Ag NPs. The thiolate conjugation chemistry can only occur with free thiols or by reducing disulfides [38]. By-products from any thiol-maleimide reaction were likely washed away in the discarded supernatant as they were unable to bind to the nanoparticle surface.

Yield values, electron-spray ionization mass spectrometry, ^{13}C and ^1H NMR analysis for the three Diels-Alder products, between 6-maleimidohexanoic acid and the furan-, thiophene-, and pyrrole-based dienes are reported in the supporting information document.

Water bath heating

The fluorescence intensities indicating FAM-siRNA release in the supernatant for all three samples, at the three different water bath temperatures are reported in Figure 2. The temperatures were selected for their physiological relevance, where higher local temperatures on nanoparticles may cause irreversible tissue damage and necrosis. The values shown were normalized to chemically reduced samples using TCEP. Normalized values were used to account for the variable particle loadings across the three different systems.

The loading efficiencies for each system was determined, based on the ratio of siRNA NP-loading to the total added siRNA. These efficiencies for the furan, pyrrole, and thiophene DA were 83.43 % $\pm$ 0.76, 77.84 % $\pm$ 1.8, and 32.89 % $\pm$ 0.24, respectively.

Based on the heating results, release of the pyrrole sample occurred most readily, even at the lowest applied temperature with 38% reversion, while release with the thiophene was not detected until 80 °C. All three DA structures were observed to fully release siRNA at 80 °C, after 2 h. The trend shown in Figure 2 agrees with the computational results above (Table 2), in which higher energy barriers were required for the reverse reaction to occur for the following reactions in specific order: thiophene > furan > pyrrole. At 60 °C, the pyrrole-maleimide cycloadduct was synthesized at a yield of 23.8 wt% (Supporting Information), but undergoes cycloreversion at the same temperature at 65% conversion rate. This was likely due to a shift in equilibrium in opposite directions in the different states. In the synthesis state, formation of the cycloadduct was aided by concentration buildup of the diene and excess maleimide, which at higher temperatures propelled the addition reaction to produce the DA linker. After conjugation to the particle, the presence of free diene and maleimide was removed, thus at the same temperature the equilibrium likely shifted further in the opposite direction to cause the reversion. Additionally, the modification of the pyrrole with the nanoparticles and siRNA molecule will likely alter the kinetics of the linker compared to its simpler diene-dienophile construct during its synthesis. While furan-based cycloadducts have always been favored due to their susceptibility to break at lower temperatures, pyrrole's superior dienofugacity over furan is often overlooked, despite the lower activation energy for cycloreversion reported by other groups [41], as well as in the current study.

With regards to the activity and stability of the siRNA exposed at these temperatures, it was determined that single stranded siRNA integrity was maintained for short incubations up to 95 °C via gel electrophoresis and functional activity assay [42]. In the current study, the thiophene-based linker required temperatures of at least 80 °C for substantial release, while the pyrrole and furan DA discharge occurred at lower and physiologically safer temperatures. It is possible at extended localized heat the siRNA may degrade if it is not timely detached, in the case of the thiophene-based linker. However in all cases, heat dissipation from the surface is expected to decrease significantly away from the immediate surface [43], and thermal stresses should swiftly be reduced as the siRNA is detached and allowed to diffuse away from the surface of the nanoparticle down its chemical potential gradient.

Second Harmonic Generation

Second harmonic generation (SHG) is a surface-sensitive technique that can be used to study the buried interfaces within solids and liquids. It is a second order non-linear spectroscopic process in which two photons of frequency ω add coherently to generate a third photon of frequency 2ω [44] [45] [46]. However, SHG is a dipole forbidden process which does not generate from centrosymmetric and isotropic bulk media but can be generated from the interfaces where the inversion symmetry is broken. There is no SHG from randomly oriented molecules in the bulk of the liquid but their attachment on the nanoparticle interfaces produces the SHG signal. The label-free probe technique of SHG spectroscopy has been

used to study oligonucleotides at nanoparticle interfaces [47] [48], photocleaving dynamics from the surface of plasmonic nanoparticles [31] [49], binding of anti-cancer drug to DNA [50], and interfacial properties of nanoparticles [51] [52] [53]. The SHG signal is induced by the second-order polarization under an incident optical electric field E_ω characterized by the second-order and third-order nonlinear susceptibilities, $\chi^{(2)}$ and $\chi^{(3)}$, respectively. The total second harmonic electric field E_{SHG} is a coherent process given by the equation [31] [49] [53] [54],

$$E_{SHG} = \sqrt{I_{SHG}} = \chi^{(2)} E_\omega E_\omega + \chi^{(3)} E_\omega E_\omega \phi_0 \quad (1)$$

where I_{SHG} is the SHG intensity and ϕ_0 is the interfacial electrostatic potential arising from the nanoparticle surface charge density in aqueous suspension. $\chi^{(2)}$ is the second-order susceptibility originating from the two-photon spectroscopy of the colloidal nanoparticle and the attached interfacial molecules. $\chi^{(3)}$ is the third-order susceptibility caused by polarized water molecules that are aligned by the static electric field near the nanoparticle surface.

The thermal release of siRNA from the surface of silver nanoparticles was studied in real time using second harmonic generation spectroscopy. Representative SHG spectra of SNP+pyrrole+maleimide+siRNA complex at 80 °C temperature are shown in Figure 3a. The SHG signal from the sample is centered at 400 nm with full width half maximum of 4.5 nm. The small rise in SHG intensity at longer wavelengths is due to two-photon fluorescence from the nanoparticle sample. The initial SHG intensity of the sample is larger due to the presence of cargo molecules on the surface of nanoparticles resulting primarily in higher $\chi^{(3)}$ contribution due to the negatively charged phosphate groups of the oligonucleotides which is consistent with our previous works [31] [49]. In fact, the use of a nucleic acid molecule as opposed to simply a fluorophore molecule results in enhancement in SHG signal that allows for the observation of a clear and distinct reduction in signal upon cleavage. The decrease in SHG intensity under increasing heating time is due to the thermal release of siRNA from the colloidal SNP surface. A control experiment showing the variation of SHG intensity as a function of heating time for SNP+pyrrole+maleimide+siRNA and SNP+cysteamine+maleimide (no diene) complex at 80 °C is shown in Figure 3c. The latter experiment was to observe the thermal effect on the stability of the thiol linkage alone to the nanoparticles. Based on Figure 3b, it can be shown that at the highest temperature treatment, no observable SHG decay representative of linker release was detected for the control sample. In **3c**, the SHG intensity from SNP+cysteamine+maleimide complex is smaller (green), as a result of the smaller surface charge density and remains constant with time. This indicates that the thiol and amide bonds are stable enough over longer periods of time at 80 °C temperature. However, the SHG intensity from SNP+pyrrole+maleimide+siRNA complex decreases with time and reaches a minimum value. This minimum value of SHG intensity is equal to the SHG intensity from SNP+cysteamine+maleimide complex, indicating a complete thermal release of siRNA from the nanoparticle interface.

Time-dependent SHG spectroscopy was used to study the thermal release of siRNA at different temperatures for the three different complexes; SNP+pyrrole+maleimide+siRNA, SNP+furan+maleimide+siRNA and SNP+thiophene+maleimide+siRNA. Figure 4a shows the variation of SHG intensity from SNP+pyrrole+maleimide+siRNA complex at 40, 60 and 80 °C temperatures. The small changes in SHG intensity (1–2 %) at 40 °C is observed for all three complexes might be due to the unavoidable small photothermal effect generated by the nanoparticle itself in the presence of the probe laser resulting in miniscule release of siRNA. To minimize this effect, a fundamental laser source having minimum power was used, the threshold to generate SHG signal. However, there were significant changes in SHG intensities at 60 and 80 °C temperatures due to the thermal release of siRNA. Similarly, the release of siRNA from the SNP+furan+maleimide+siRNA and SNP+thiophene+maleimide+siRNA complexes at the three different temperatures are shown in Figure 4b and 4c respectively.

The thermal-cleaving rate of siRNA was shown to be maximum in the pyrrole-based cycloadduct and minimum in the case of the thiophene cycloadduct chemistry. The thermal-cleaving rate constants at different temperatures were obtained by fitting the SHG data using a pseudo-first order exponential rate equation, as described in previous works [31] [49]. The different saturation levels reached at 60 and 80 °C for each rDA reaction indicates the likelihood that endo isomers, typically more sterically hindered and hence easier to reform into their corresponding reagents [23], are triggered at lower temperatures while the more stable exo isomers are released at the higher temperature. The endo isomer is usually thermodynamically favored to form in the Diels-Alder reaction, and is often referred to as Alder's rule [55]. In the reaction between cyclopentadiene and maleic anhydride the product formed is in the endo isomer form at room temperature but forms the exo isomer at high temperatures as the latter has fewer sterically repulsive interactions [56]. The rate constants obtained for the three different complexes at three different temperatures are summarized in Table 3.

Light activated photothermal release

The SNP samples conjugated with the three different DA linkers were irradiated at 405 nm, a wavelength that overlaps with the SNP plasmon resonance (Figure S1). Localized heating of the nanoparticle induced cycloreversion of the DA linkers, with varying rates based on the demonstrated kinetics previously illustrated. The normalized release profiles are shown in Figure 5. All the samples were normalized to the total oligo on the surface of the nanoparticle, represented by sulfide-reducing a non-irradiated control sample. The patterns show a similar trend in which faster photothermal release is observed with the pyrrole-based sample, while a much slower decay is observed with the thiophene sample. Localized heating in plasmonic nanoparticles can be explained by the metal's ability to absorb far field radiation and produce enhanced near fields [57]. Plasmonic resonances decay by re-emission of an electron or creation of hot electron-hole pairs by Landau damping [58]. For plasmonic energies greater than the interband transition threshold, hot holes will mainly form at the upper edge of the noble metal d band with the electrons located at the thermal Fermi energy level or higher [59]. Hot electrons will then re-distribute that energy to lower energy electrons via electron-electron scattering until equilibrium is reached and heat is dissipated

from the lattice to the surroundings. The choice of silver nanoparticles was based on their utility in biological applications, a result of their non-toxicity to human cells and their anti-microbial and anti-fungal properties [60]. Optically, they exhibit a large absorption cross section [61], suggesting a more efficient light-to-heat conversion response [62]. This localized heat phenomena can be utilized to generate adequate thermal energy and induce the rDA reaction. Bulk solution temperatures were measured and reached a steady state of 48 °C, as shown in Figure S2. The trends in Figure 5 highlight the impactful use these linkers possess to achieve sequential gene delivery with enhanced temporal control utilizing plasmonic metal nanoparticles. For example, by altering exposure time to light stimulation delivery of three different nucleic acid cargo can be potentially achieved as a result of the varying thermal kinetics of the linker conjugation and their respective rDA initiation. Furthermore, varying doses can be achieved by altering the delivered light energy with a more rapid release observed with the pyrrole DA linker, and a more sustained and prolonged release with the thiophene DA.

The residual groups on the silver nanoparticles after the rDA reaction will be the heterocyclic dienes. Furan is listed as a carcinogenic substance by the FDA and Department of Health and Human Services Report on Carcinogens, based on animal tests, and is considered possibly carcinogenic to humans by the International Agency for Research on Cancer. However, it was recently published by the FDA that intake of furan-contaminated foods averaged 15 µg/day [63] but was still deemed safe for consumption. Our delivery system will contain less than 1µg of the furan, depending on dosages of therapeutic in future studies. Meanwhile, toxicity information on pyrrole-based drugs is scarce, but was recently shown to have acute effects on blood glucose levels with no significant morbidity impacts on the studied rat models [64]. Of the three linkers, thiophene shows the most possible potential for induced toxicity. Biotransformation of heterocyclic rings present in drugs have raised concerns, and of them, thiophene was declared as a structural alert [65] [66]; its metabolism forms reactive metabolites, which are responsible for drug-induced hepatotoxicity. Unsubstituted thiophene has reported LD50 values of 1902 and > 500 mg/kg body weight in mice [67]. At this stage it is difficult to assess accurate toxicology responses of the residual dienes, given their conjugated nature, at the given dosages. Further studies involving *in vitro* and *in vivo* applications of these DDSs will determine their respective toxicities which will hopefully be showcased in future manuscripts.

Additionally, the presence of the maleimide on the 3' terminal of the siRNA sequence may impact the activity of the siRNA. Previous studies indicate that siRNA retain RNAi activity after 3' modification with several different chemical moieties [68] [69] [70], but at this time, it is not clear what effect, if any, the 3' maleimide modification has on the siRNA's ability to interact with the 3' UTR region of mRNA. Further studies are required to address this concern.

Conclusion

The above results demonstrate predictable thermal and photothermal responses for three rDA compositions. The heated pyrrole-based DA cycloadduct had the most rapid release of the siRNA, with entire release occurring within minutes under excitation. Both the furan and

thiophene linkers required several minutes to initiate the rDA reaction, with the thiophene in particular showing prolonged stability at high temperature. While furan-based DA cycloadditions are considered the gold standard in the field of drug delivery systems, here we have utilized the additional chemistries of pyrrole-based and thiophene-based bicyclic products with demonstrated breaking responses. Based on the kinetic information gained here from SHG spectroscopy and other analytical methods, the retro-Diels-Alder reaction can be utilized as a powerful tool to temporally release genes, with alternate chemistries generating a range of temperature trigger zones to initiate cleavage. Employing nano-vehicles with localized heat generation abilities, such as plasmonic or magnetic particles, temperatures high enough to release cargo but also limit thermal degradation to surrounding tissue can be achieved for sequential gene delivery. Application of these linkers as effective therapeutic delivery systems will need to be demonstrated in future *in vitro* and *in vivo* studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Overall schematic illustrating pericyclic reaction between dienes with 6-maleimido-hexanoic acid and conjugation onto nanoparticle via generic thiol linkage (1). EDC coupling chemistry was utilized to link amine-terminated siRNA to nanostructure (2).

Figure 2. Normalized fluorescence of FAM-siRNA release from covalently-linked SNPs via retro-Diels-Alder reaction at different water bath temperatures, $\pm$ SEM.

Figure 3.

a) SHG spectra for SNP+pyrrole+maleimide+siRNA sample at 0, 30, and 300 s, b) normalized SHG spectra for control sample, SNP+cysteamine+maleimide, c) and real time decay of SHG maxima at 400 nm for SNP+pyrrole+maleimide+siRNA and SNP+cysteamine+maleimide linker, $\pm$ SEM.

Figure 4. Time-dependent SHG profile of thermal release of siRNA from (a) SNP+pyrrole+maleimide +siRNA, (b) SNP+ furan+maleimide+siRNA and (c) SNP+thiophene+maleimide+siRNA complexes, +/-SEM.

Figure 5. Photothermal release of SNPs conjugated with the 3 different DA linkers, after 405 nm resonant irradiation, measured with fluorescence intensity, $\pm$ SEM.

Table 1

Optimized structures used for B3LYP/ 6-311G* computation. Yellow=S, Red=O, Black=C, Blue=N, White=H. Images rendered using PyMOL [33].

Diene	Maleimide	Transition State	Product
2-furanmethanethiol

2-thienylmethanethiol

Pyrrole-2-carboxylic acid + cysteamine

Table 2

Gibbs free energy & enthalpy reaction barriers generated from B3LYP/6-311G* theory.

Diene	Reaction	Reaction Barriers for H_{rxn}				Reaction Barriers for G			
		kcal/mol				kcal/mol			
		25 °C	40 °C	60 °C	80 °C	25 °C	40 °C	60 °C	80 °C
2-furanmethanethiol	Forward	21.32	21.33	21.34	21.36	35.56	36.28	37.23	38.18
	Reverse	23.15	23.17	23.19	23.21	21.82	21.75	21.66	21.57
2-thienylmethanethiol	Forward	28.66	28.67	28.67	28.68	43.88	44.65	45.67	46.69
	Reverse	30.42	30.43	30.45	30.47	29.32	29.26	29.19	29.11
Pyrrole-2-carboxylic acid + cysteamine	Forward	23.86	23.86	23.86	23.87	39.14	39.91	40.93	41.96
	Reverse	18.98	19.01	19.04	19.08	17.53	17.45	17.35	17.25

Table 3

List of fit parameters obtained by fitting SHG data using first order exponential equation, $\pm$ SEM.

Temperature (°C)	Rate Constant ($\text{s}^{-1} \times 10^{-2}$)		
	Pyrrole	Furan	Thiophene
40	0.056 ± 0.020	0.037 ± 0.008	0.021 ± 0.013
60	1.426 ± 0.109	0.304 ± 0.055	0.182 ± 0.105
80	2.650 ± 0.119	0.345 ± 0.036	0.227 ± 0.053