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Two-Photon-Induced \([2 + 2]\) Cycloaddition of Bis-thymines: A Biocompatible and Reversible Approach

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ABSTRACT: Despite having great value across a wide variety of scientific fields, two-photon polymerizations currently suffer from two significant problems: the need for photoinitiators, which generate toxic side products, and the irreversibility of the process. Hence, the design of a versatile approach that circumvents these issues represents a major scientific challenge. Herein, we report a two-photon absorption strategy where reversible \([2 + 2]\) cycloaddition of bis-thymines was achieved without the need for any photoinitiator. The cycloaddition and cycloreversion reactions could be induced by simply changing the irradiation wavelength, and repeated writing and erasing cycles were performed. The simplicity, reversibility, and biocompatibility of this strategy open up a whole new toolbox for applications across a wide variety of scientific fields.

INTRODUCTION

Two-photon (TP) excitation was theoretically predicted in 1930,\textsuperscript{1} empirically demonstrated 30 years later,\textsuperscript{2} and transformed the future of material science thereafter.\textsuperscript{3} It is defined as the simultaneous absorption of two photons, each carrying nearly half of the energy required to excite a molecule to higher energy states.\textsuperscript{4,5} Its success relies on two key ingredients. First, the two-photon absorption depends quadratically on the incident light intensity, thus sharply decreasing outside the focal volume. As a consequence, any TP-induced polymerization can be confined in a small region of the sample by tightly focusing the excitation light. This way, different patterns can be drawn,\textsuperscript{6} and potentially erased, and the material refractive index can be changed only in the irradiated zones.\textsuperscript{7} Optical storage devices and optical gratings, based on direct laser writing, represent a few examples of possible applications.\textsuperscript{8} Second, TP-induced reactions use long-wavelength light such as near-infrared, which confers a significant benefit in biological applications, where the phototoxicity is significantly reduced and extended penetration depths can be achieved.\textsuperscript{9,10} These features, combined with the high spatial confinement, result in an exceptional cocktail, which enables the use of TP-based polymer particles for the controlled release of biomolecules in vivo.\textsuperscript{11,12} As a result, a considerable volume of research has been carried out in the field of TP-induced polymerization, and significant achievements have been reached hitherto.\textsuperscript{13} However, existing TP-based polymerization schemes still suffer from a few significant pitfalls. The chemical bonds formed during the reactions are typically stable, rendering the process irreversible.\textsuperscript{14} In addition, they normally require the use of photoinitiators to trigger the reactions, which generate radicals that create unwanted and highly (cyto)toxic side products, such as reactive oxygen species.\textsuperscript{15,16} To achieve a universal platform for TP-induced reactions, it is crucial to develop a versatile approach that overcomes these issues.

\([2 + 2]\) Cycloaddition reactions represent promising candidates to be studied by TP absorption, thanks to the significant control over the stereochemistry of the polymer chains and the eco-friendly hallmarks.\textsuperscript{17} So far, few TP-induced reactions based on \([2 + 2]\) cycloaddition have been designed, which involve polymers containing coumarin and maleimide side chains. Here, however, photoinitiators are still required and complete reversibility remains a challenge.\textsuperscript{18,19} To knock over these barriers, we report a two-photon polymerization strategy, where different chemical structures can be easily obtained by only changing the irradiation wavelength and without the need of any photoinitiator.

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We previously designed a bis-thymine monomer, \( n \)-butyl-linked-bis(thymine propanoate), which can undergo one-photon-induced [2 + 2] cycloaddition and cycloreversion upon irradiation with UV light in the solid crystalline state.\(^{20−22}\) Here, we demonstrate that the same reaction could be achieved upon TP absorption (Figure 1).

![Figure 1. [2 + 2] Cycloaddition and cycloreversion reactions of \( n \)-butyl-linked-bis(thymine propanoate). The reacting moieties are highlighted in green and blue, while the red dashed lines indicate the bond formation pathway.](image)

**RESULTS AND DISCUSSION**

**Cycloaddition.** In the first stage, we investigated whether the bis-thymine monomer could undergo cycloaddition upon TP absorption. In a typical experiment, a monomer crystal was irradiated with 600 nm focused light (1.6 mW, 1 min, 200 fs, 80 MHz). As depicted in Figure 2a, changes in the crystal occurred after irradiation (round spots) and are provoked either by a modification in the material refractive index or by a crack generated in the crystal. Such alteration is, in turn, caused by the strain imparted on the crystal lattice upon reorientation of the molecules, as reported by previous studies.\(^{20}\)

To investigate which product resulted from the irradiation, we initially conducted NMR measurements, which unfortunately resulted in no signal due to the low amount of sample. Consequently, we used Raman spectroscopy, as its sensitivity allows to analyze reactants at sub-micrometer scale. Not to mention, Raman spectroscopy represents an ideal candidate in chemical reaction studies, thanks to its remarkable fingerprinting capabilities, which provide chemical and structural information unique for each species. Particularly, [2 + 2] cycloaddition reactions represent straightforward subjects of study, as the bis-thymine and cyclobutane structures give distinct Raman fingerprints.\(^{23−25}\) Investigations through Raman spectroscopy require reference spectra of the compounds under study, which are compared with the spectra obtained during the reactions. In the light of this finding, the Raman spectra, corresponding to the bis-thymine molecules and photoinduced cyclobutane samples synthesized via one-photon reaction, were acquired and kept as a reference throughout all this work. An extensive assignment of the vibrational bands is available in the Supporting Information (SI) (Figure S2). The Raman spectra collected from the TP-induced spots display bands associated with the skeletal stretching vibrations of cyclobutane ring at 498, 707, and 922 cm\(^{-1}\) (Figure 2c, vertical dotted lines) and are in good agreement with the reference spectrum. The Pearson correlation coefficient (PCC), which can be regarded as a measure of similarity, was calculated between the reference and extracted spectra (more information in SI). The obtained value of PCC is 0.84, which indicates a high correlation, thus confirming the successful TP-induced cycloaddition.

![Figure 2. Cycloaddition and cycloreversion reactions of bis-thymines induced via two-photon excitation. (a) Transmission image of the bis-thymine monomer crystal after inducing cycloaddition in three spots. (b) Raman mapping of a sample area, featuring the monomer (green) and the cyclobutane (blue). (c) Raman spectrum of the cyclobutane structure compared with the reference spectrum. (d) Transmission image recorded after erasing one spot of previously photoinduced cyclobutane. (e) Raman mapping of the same sample area, confirming the complete cycloreversion. (f) Raman spectrum of the obtained monomer compared with the reference spectrum. Scale bar: 5 μm.](image)
Small variations in the vibrational bands between the one-photon (reference) and TP-generated samples (photoinduced) were, however, noticeable, and were due to the difference in crystallinity (Figure S3).

Notably, we discovered that the cycloaddition yield strongly depends on the crystal orientation with respect to the light polarization (Figure S4). We hypothesize that such orientation dependence arises from the anisotropic nature of the crystals, in which molecules are packed unidirectionally.20,26

Next, we aimed at evaluating the confinement volume of the reaction, and Raman mapping represents an excellent approach to assess it. Hence, a designated area in the sample (Figure 2a, red rectangle) was scanned with a laser and Raman spectra were collected point-by-point. A false-color image was then generated from the peak intensities corresponding to the cyclobutane stretching vibration (blue 922 cm\(^{-1}\)) and the thymine deformations (green 1638, 1658 cm\(^{-1}\)) (Figure 2b). The image obtained reveals that the cycloaddition is limited to the irradiated spots.

Figure 3. Power dependence measurements. (a) Raman spectra collected after irradiation with different femtosecond laser powers. (b) Intensity of the peak associated with the cyclobutane structure (vertical dotted line) against the laser power. Both ordinate and abscissa are given as logarithmic scales.

We then confirmed the TP nature of this process. The UV–vis spectra recorded from the reference samples show no absorption in the spectral region above 300 nm, excluding that the reactions take place via one-photon absorption (Figure S5). The typical evidence for TP process is given by power dependence investigations as, in a TP process, the absorption exhibits quadratic dependence on the irradiation power. Consequently, different spots in the monomer crystal were irradiated with various laser powers, and after each irradiation step, a Raman spectrum was collected (Figure 3a). The peak intensity corresponding to the cyclobutane ring vibration at 922 cm\(^{-1}\) was plotted as a function of the laser power (Figure 3b). The scattered data were fitted with a power function, \(I = P^a\), where \(I\) is the peak intensity, \(P\) is the laser power, and \(a\) is the power of the function. From this fitting, the estimated value was 1.75, confirming the occurrence of TP absorption. The deviation from the predicted value 2 can be attributed to the saturation of polymer product within the 600 nm irradiation spot.27

Cycloreversion. Cycloreversion reactions were investigated in an entirely analogous fashion. Accordingly, one spot of the previously photoinduced cyclobutane was irradiated with 450 nm pulsed light (0.3 mW, 1 min, 200 fs, 80 MHz). The irradiated spot could be erased, and the monomer crystal completely regenerated (Figure 2d). This result suggests that the spots originating after 600 nm irradiation (Figure 2a) were caused by a change in the refractive index and not by cracks formed in the crystal. Following the same approach as outlined for the cycloaddition, we collected the Raman spectra corresponding to the irradiated area and compared them with the Raman fingerprint of the original monomer (Figure 2f, PCC value: 0.98). In the spectrum collected from the photoinduced spot, bands related to the cyclobutane ring vanished entirely, while peaks attributed to the thymine moieties at 447, 459, 507, 521, 1638, and 1656 cm\(^{-1}\) re-emerged (Figure 2f, red dotted lines). These spectral changes suggest the successful cleavage of the cyclobutane rings, as well as the formation of the initial bis-thymine units. Once again, Raman mapping in the same area was carried out (Figure 2e). The image obtained further confirms the loss of the cyclobutane structures.

In addition, we investigated the wavelength dependence of the process, as the irradiation wavelength appears to have a strong influence on the reaction products. Surprisingly, we discovered that when 500 nm light was employed instead of 450 nm light, a different compound was formed (Figure S6). We hypothesize that a partial cycloreversion occurred via charge separation, a mechanism previously shown by Tsurkan et al. in \([2 + 2]\) cycloaddition reactions.19 A more in-depth investigation of such a new structure is beyond the purpose of this work, although it deserves future investigations.

Cycloaddition and Cycloreversion Cycles. The last set of experiments was aimed at exploring whether our compounds could undergo repeated cycles of cycloaddition and cycloreversion. The same spot in the monomer crystal was irradiated repeatedly by alternating 600 and 450 nm light. As revealed by the resulting Raman spectra, the monomer and cyclobutane structures could be obtained after each irradiation step (Figure 4a). To better visualize this achievement, peak intensities related to the bis-thymine (1638 cm\(^{-1}\)) and the cyclobutane (922 cm\(^{-1}\)) were plotted against the irradiation step (Figure 4b).

CONCLUSIONS

Although two-photon polymerizations are in the spotlight of an ever-growing research endeavor, limitations related to the need for photoinitiators and the irreversibility of the process have not been overcome, yet. In this work, two-photon-induced \([2 + 2]\) cycloaddition of bis-thymine monomers was achieved without any photoinitiator and in a fully reversible fashion. The key to the success of this strategy lies in its versatility. The reactions can be induced by only varying the irradiation wavelength, rendering this approach easy to perform. The complete reversibility of the process enables
repeated writing/erasing/rewriting cycles, along with repeated modifications of the material refractive index. Moreover, the absence of photoinitiators renders this material biocompatible, suggesting potential for its implementation in biomedical applications.

- **EXPERIMENTAL SECTION**

**Sample Preparation.** Thymine-based compounds were synthesized and characterized by the group of Prof. K. Saito following a previously described protocol.\(^2^2\) The monomer powder was dissolved in a 50/50 (v/v) mixture of acetonitrile/H\(_2\)O (10 mg·mL\(^{-1}\), 21 \(\mu\)M). The solution was vortexed to ensure that the whole monomer was dissolved and 70 \(\mu\)L was drop-casted on a clean glass coverslip. The solvent was allowed to evaporate overnight to yield white crystals, which were attached to the glass coverslip (Figure S1). A single-photon mixture of chloroform/H\(_2\)O (10 mg·mL\(^{-1}\)) was synthesized polymer was either dissolved in a 50/50 (v/v) mixture of acetonitrile/H\(_2\)O (10 mg·mL\(^{-1}\)) to yield a filmlike morphology, or in a 50/50 (v/v) mixture of acetonitrile/H\(_2\)O (10 mg·mL\(^{-1}\)) to yield a crystal-like morphology. Both solutions were then drop-casted onto a glass coverslip following the same procedure as described for the monomer. After the evaporation of the solvent, film and crystals structures appeared (Figure S3).

**Two-Photon Reaction.** The two-photon experiments were performed on the same setup as that used for Raman spectroscopy. A glass coverslip containing either a monomer or a polymer was placed on the piezoelectric stage of the microscope. A Ti:sapphire femtosecond laser (MaiTai, Spectra-Physics, 820 nm, 120 fs, 80 MHz) coupled with an optical parametric oscillator (OPO) (Inspire, Spectra-Physics) was used to produce femtosecond pulses (80 MHz, 120 fs, linearly polarized) to induce two-photon reactions. The light was deflected by a dichroic mirror (Z633RDC, Chroma) before being focused on the sample by the same objective as that used for Raman spectroscopy. Three different wavelengths were used, i.e., 600 nm for cycloaddition (1.6 mW), 450 nm for cycloreversion (0.3 mW), and 500 nm for obtaining the unknown compound (1 mW). For a typical experiment, an irradiation time of 1 min was used.

**Raman Spectroscopy.** Raman measurements were conducted using an inverted optical microscope (Ti–U, Nikon) equipped with a piezolectric stage (PS173CL, Physik Instrument). Continuous-wave He–Ne laser light (632.8 nm) was reflected by a dichroic mirror (Z633RDC, Chroma) and then focused on the sample by an objective lens (Plan Apo 60x, N.A. 0.95 or Plan Fluor 40x, N.A. 0.60, Nikon). Circular polarization at the sample was achieved by tuning half-wavelength (\(\lambda/2\)) and quarter-wavelength (\(\lambda/4\)) waveplates. The Raman signals were collected by a spectrometer (iHR 320, Horiba) equipped with a cooled electron-multiplying charge-coupled device (CCD) camera (Newton 920, Andor).

**Pearson Correlation Coefficient (PCC).** The Pearson correlation coefficient (PCC) between the two spectra was estimated according to the following equation

\[
 r_{xy} = \frac{\sum_{n=1}^{N}(x_n - \bar{x})(y_n - \bar{y})}{\sqrt{\sum_{n=1}^{N}(x_n - \bar{x})^2 \sum_{n=1}^{N}(y_n - \bar{y})^2}}
\]

where \(N\) is the total number of considered Raman shifts; \(x_n\) and \(y_n\) are the Raman intensities registered for the first and second spectra, respectively, at the \(n\)th Raman shift; and \(\bar{x}\) and \(\bar{y}\) are the mean intensities recorded for the first and second spectra, respectively. \(r_{xy}\) can be regarded as a measure of the overall similarity between the two profiles being studied. Even though there exists no widespread consensus on the interpretation of the PCC, the coefficients calculated in this work reflect relatively high correlations given the noise level of the measurements and the high amount of experimental factors influencing them. To obtain an unbiased estimation of the PCC, each pair of spectra was preliminarily baseline-corrected by asymmetric least squares (AsLS).\(^2^8\)

- **ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00770.

Experimental procedures; Raman characterization and peak assignment; morphology dependence; orientation dependence; UV–vis spectra; and reaction with 500 nm light (PDF)

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[Figure 4](#) Repeated cycles of cycloaddition and cycloreversion of bis-thymines. (a) Raman spectra collected from the same spot after alternate irradiation with 600 and 450 nm light. (b) Peak intensities associated with the thymine (green bands) and cyclobutane (blue bands) against the irradiation cycles.
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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
TP, two-photon; NMR, nuclear magnetic resonance; PCC, Pearson correlation coefficient

REFERENCES
