Abstract: N-acyl-7-nitroindolines have been used as caged compounds to photorelease active molecules by a one- or two-photon excitation mechanism in biological systems. Here, we report the photolysis of a polypeptide that contains 7-nitroindoline units as linker moieties in its peptide backbone for potential materials engineering applications. Upon two-photon excitation with femtosecond laser light at 710 nm the photoreactive amide bond in N-peptidyl-7-nitroindolines is cleaved rendering short peptide fragments. Thus, this photochemical process changes the molecular composition at the laser focal volume. Gel modifications of this peptide can potentially be used for three-dimensional microstructure fabrication.

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References and links

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1. Introduction

Photoremovable protecting groups (PPGs) are required in “caged compounds” in which the function of the original compound is inhibited. Upon photo excitation PPGs are removed and the function of the compound is restored. Such PPGs include arylcarbonylmethyl, nitrobenzyl, nitroindolinyl, and their derivatives, amongst others [1]. Currently the most common application of this photolysis process is the spatially and temporally controlled release (“uncaging”) of bioactive molecules such as neurotransmitters, carboxylic acids, ATP, Ca\(^{2+}\) ions, fragrances, etc [2, 3]. Uncaging can also be used for the photochemical conversion of weakly or non-fluorescent molecules into strongly fluorescent ones [4]. In each of the above applications, the purpose of inducing photolysis is to release compounds with the desired bioactivity or physical property. Here, we explore a new use of photolysis for the potential fabrication of new materials that may serve as scaffolds or matrices for tissue engineering. Within a macroscopic gel-like material bonds may be cleaved only at the light illuminated locations. The molecular fragments generated by photolysis (Fig. 1(a)) are no longer of interest; and upon their removal from the macroscopic material, three-dimensional structures may be left behind. This approach can potentially achieve similar results as two-photon polymerization based microfabrication [5], albeit by a different mechanism. In order to test such an approach in the future, a three-dimensional network of a material that can be manipulated by two-photon photolysis is essential. For example, a gel-forming peptide with certain strategically placed, photoreactive groups (Fig. 1(a)) could potentially serve as a source for such a material.

We have synthesized a peptide that resembles collagen in terms of amino acid composition with four photocleavable 7-nitroindoline moieties built into the peptide backbone, peptide 1 (Fig. 1(b)) [6]. Collagen mimicking peptides (CMPs) are commonly used materials in tissue engineering to mimic either structural or functional characteristics of natural collagens which aims at engineering higher order structures similar to natural tissue scaffolds [7]. When compared to natural collagen, the benefits of using CMPs include the ability for customization as well as reversible melting behavior with complete efficiency once the CMP is cooled due to its small size [7].

![Fig. 1](image-url)  
Fig. 1. (a) A polypeptide with built-in photoreactive moieties (red) may undergo photolysis at all photoreactive sites when irradiated with a femtosecond laser at 710 nm. This photolysis generates a number of small peptide fragments. (b) Molecular structure of peptide 1: 34-mer peptide with four photoreactive N-peptidyl-7-nitroindoline units (red), which themselves can be regarded as amino acids.
N-acyl-nitroindoline based PPGs typically have broad absorption spectra in the wavelength range shorter than 500 nm [8, 9]. Based on the success of two-photon uncaging of a methoxy derivative of nitroindolino glutamate [10], we explored the feasibility of using an in-house developed two-photon microscope to cleave the amide bonds of the N-peptidyl-7-nitroindoline units within the newly synthesized peptide 1 [11]. Two-photon absorption has the advantage of confined spatial excitation at the focal volume due to its nonlinearity characteristic. It is also convenient to incorporate two-photon absorption based photolysis into commonly used two-photon fluorescence microscopes to achieve high spatiotemporal resolution for microfabrication. In addition, the fluorescence decay of N-acyl-7-nitroindolines can be measured with these imaging microscopes.

2. Synthesis

The detailed synthesis of the photoreactive peptide 1 will be published elsewhere [6]. Briefly, the peptide was assembled by on-resin fragment condensation using Fmoc/tert-Bu strategy solid phase peptide synthesis (SPPS). The C-terminal hexapeptide was synthesized on Rink amide resin and elongated four times with the photoreactive hexapeptide, which had been synthesized by SPPS using diphenyldiazomethane resin and a photoreactive glycine building block [12, 13] under standard coupling and deprotection conditions (Fig. 2). The crude polypeptide was purified by reversed phase Fast Protein Liquid Chromatography and lyophilized.

3. Two-photon microscope

The details of our in-housed developed two-photon microscope was described in Reference [11]. In summary the light source is a mode-locked Ti:Sapphire laser (Maitai HP, 690-1040 nm, 100 fs, 80 MHz, Newport, Santa Clara, CA). We have used 710 nm light to achieve two-photon excitation of N-acyl-nitroindoline. The home-built x-y scanner (polygon, galvanometer) can achieve 30 frames/s scanning rate. The laser power at the sample site is varied by rotating a half-wave plate in front of a polarizer. The fluorescence signal from the sample are detected in three spectral channels with photomultiplier tubes (PMTs): red (570-616 nm), green (500-550 nm), and blue (417-477 nm). The outputs of these three PMTs are fed into red/green/blue channels of a frame grabber (Solios eA/XA, Matrox, Quebec, Canada). Two-dimensional images in the x-y plane are acquired through a home-built software program. Each frame has 500 × 500 pixels. Each final static image is an average of 30 frames.

4. Results

The lyophilized peptide 1 (~1 mg) was dissolved in 2 μL of water on a microscope slide to give a yellow (~125 mM) solution. At this concentration, peptide 1 quickly forms a gel. The film/gel was covered with a cover slip (Fig. 3(a)).
Several spots within the sample were irradiated under the two-photon microscope using 710 nm light with varying excitation laser output powers ranging from 100 to 200 mW with increments of 25 mW. The delivered laser power at the sample location is 10% of such values. Upon excitation, the photoreactive peptide 1 emits fluorescent light which is collected using both red and green PMTs. This excitation also induces photolysis of the amide bond between glycine and 7-nitroindoline, producing non-fluorescent nitroindoline and/or 7-nitrosoindole derivatives (brown spots in Fig. 3(b)), which are darker in color than the N-acylated nitroindoline precursor 1. Therefore, as the photolysis reaction progresses, the fluorescent peptide 1 is consumed, and consequently a decrease in average fluorescence intensity at the irradiation site is observed. For each irradiated spot, a time series of fluorescence images was recorded at every minute to track the fluorescence intensity decay throughout the photo induced reaction process (Fig. 4(a)-4(d)). Once the fluorescence decay appeared to reach a plateau, the laser irradiation at this spot was stopped and a new location was chosen to repeat the process with a different laser power. To quantify fluorescence decay a defined region of interest was chosen within the image, and the average fluorescence intensity in each of the green and red channels was measured. This measurement was
repeated for the same region of interest for every image in each time series. These fluorescence decay curves from a single sample of peptide 1 are shown in Fig. 4(e).

These fluorescence decay data were fitted using an exponential decay regression line of the form $F(t) = F_0 e^{-\beta t}$, where $\beta$ is the fluorescence decay rate. This was done using the curve fitting module in MATLAB. As mentioned before, the decrease in fluorescence intensity correlates with the photolytic reaction of the compound under the incident light. Therefore, the fluorescence decay rate $\beta$ is also the photolysis reaction rate within the focus of the sample. Since the photolysis is occurring as a result of two-photon absorption, the photolysis reaction rate is proportional to the probability of two-photon absorption as shown in Eq. (1) where $I$ is the excitation laser power

$$\beta \propto I^2$$

Plotting $\log(I)$ versus $\log(\beta)$ for each of the laser intensities and their corresponding fluorescence decay rates produces a linear graph whose slope should be 2 for a two-photon process. Therefore, the photochemical reaction rate’s quadratic dependence on laser intensity may easily be evaluated using a double-log plot. The slope of the regression line in the double-log plot in Fig. 4(f) is 2.007, which clearly exhibits the two-photon absorption induced nature of the photolysis within the synthetic 34-mer peptide 1.

Fig. 5. High Resolution Electrospray Ionization-Time of Flight mass spectrum of the crude mixture obtained after irradiation of peptide 1 (sample in Fig. 3(b)). Reported are the monoisotopic masses for each peptide. 1 (C$_{156}$H$_{197}$N$_{39}$O$_{47}$): m/Z for [M + 4H]$^{4+}$ calc. 843.1134, found 843.1198; m/Z for [M + 3H]$^{3+}$ calc. 1123.8153, found 1123.8219; m/Z for [M + 2H + Na]$^{2+}$ calc. 1131.1426, found 1131.1436; m/Z for [M + H + 2Na]$^{4+}$ calc. 1138.4699, found 1138.4693; m/Z for [M + 2H]$^{2+}$ calc. 1685.2190, found 1685.2159; m/Z for [M + H + Na]$^{2+}$ calc. 1696.2100, found 1696.2161. 2 (C$_{66}$H$_{82}$N$_{16}$O$_{21}$): m/Z for [M + H]$^{+}$ calc. 1245.5488, found 1245.5488; m/Z for [M + Na]$^{+}$ calc. 1457.5810, found 1457.5810. 5 (C$_{66}$H$_{83}$N$_{17}$O$_{20}$): m/Z for [M + H]$^{+}$ calc. 1435.5919, found 1435.5928; m/Z for [M + Na]$^{+}$ calc. 1457.5738, found 1457.5810. 6 (C$_{57}$H$_{76}$N$_{14}$O$_{18}$): m/Z for [M + H]$^{+}$ calc. 1245.5488, found 1245.5488; m/Z for [M + 2H]$^{2+}$ calc. 1071.9612, found 1071.9612. 9 (C$_{90}$H$_{116}$N$_{22}$O$_{28}$): m/Z for [M + 2H]$^{2+}$ calc. 977.4326, found 977.4326. 10 (C$_{132}$H$_{163}$N$_{33}$O$_{40}$): m/Z for [M + 2H]$^{2+}$ calc. 1426.0964, found 1426.0964. 11 (C$_{123}$H$_{156}$N$_{30}$O$_{38}$): m/Z for [M + 2H]$^{2+}$ calc. 1331.5742, found 1331.5742.
Analysis of the mass spectrum (Fig. 5) of the crude sample post-irradiation (Fig. 3(b)) revealed the presence of all eleven expected linear nitroindoline-containing peptide fragments of various lengths (2 – 11, Fig. 6). Since each molecule contains four photocleavable sites, some molecules may undergo fewer than four photolytic reactions during the short irradiation period. Therefore, it is not surprising to have identified many incompletely photolyzed peptides (5 – 11) in the crude mixture. Since only a small fraction of the peptide sample was irradiated, the full length peptide 1 is the major component found in the mass spectrum (Fig. 5). Although the mass spectrum of the crude mixture also contains several unidentified signals, there is no evidence of any cross-linking between peptides.

A further indication for the occurrence of photolysis within irradiation sites is the color change of the sample. The original peptide 1 is bright yellow in color and its color remains the same after being dissolved in water at high concentration. In contrast, the peptide fragments have a dark brown color due to nitroindoline derivatives that are no longer acylated. The color is noticeably different from the original peptide 1 upon visual inspection by comparing Fig. 3(a) vs. 3(b). The positions of dark brown spots formed within the sample match the pre-recorded irradiation locations (Fig. 3(b)) which, together with the double-log plot of reaction rate vs. laser intensity (Fig. 4(f)), indicates the two-photon absorption induced photochemical cleavage.

5. Conclusions
We have studied the ability of a new collagen resembling peptide 1, composed of five Pro-Pro-Gly-Hyp-Pro-Gly hexamers covalently linked together by four 7-nitroindoline groups to undergo two-photon photolysis. Peptide 1 contains four N-peptidyl-7-nitroindoline moieties that are fluorescent and photoreactive. Femtosecond laser induced fluorescence decay experiments show that these N-peptidyl-7-nitroindoline moieties can be cleaved photolytically. The double-log plot of reaction rate vs. laser intensity has a slope of 2, which proves that the photolysis occurred through a two-photon absorption process. This new type of photoreactive material lays the foundation for future research on fabricating three-dimensional microstructures.

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