Two-Photon Photolysis of 2-Nitrobenzaldehyde Monitored by Fluorescent-Labeled Nanocapsules

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In this paper, we report, for the first time, experimental evidence of multiphoton photolysis of a caged proton compound, 2-nitrobenzaldehyde (o-NBA), using a new sensor system that utilizes fluorescent-labeled nanocapsules, i.e., a fluorescent nanostructured shell of micrometric size and nanometric thickness. The photolabile compound undergoes one-photon absorption in the UV range (200–380 nm), and the mechanism that leads to proton release is based on the well-known 2-nitrobenzyl photochemistry, which has been used for many photoactivatable-caged compounds. Because the use of UV excitation can cause biological damage, we changed our focus to multiphoton absorption—uncaging processes. The induced pH decrease was monitored by imaging changes in the pH-dependent emission of fluorescein isothiocyanate that was embedded in a nanostructured system (so-called “nanocapsules”). The nanocapsules with covalently bound dyes allow improved stability in fluorescence monitoring. Moreover, an original image processing method is introduced to quantify the uncaging. Using a femtosecond Ti:sapphire laser that was operating at 720 nm, with a pulse width of ~200 fs at the sample, delivered through an adapted confocal laser scanning head and a 1-min exposure time with high power (45–50 mW), we obtained appreciable photolysis of 2-nitrobenzaldehyde. So far, we demonstrated that fluorescent-labeled nanocapsules are a suitable system as fluorescence sensors.

Introduction

The expanding interest in photoactivatable caged compounds for biological applications is leading, on one hand, to the development of new classes of caged effectors and photosensitive molecules and, on the other hand, to new experimental techniques for in vivo uncaging.

One of the largest classes of caged molecules that has been exploited so far is based on the photochemistry of 2-nitrobenzyl chromophores. This classification is comprised of an impressive number of caged effectors and photolabile parent compounds. One of the crucial problems in the uncaging process is that the photolysis requires high-energy beams. The use of high-intensity UV excitation is sometimes not compatible with biological samples, because it causes damage; therefore, it is of the greatest interest to exploit the photolysis capabilities under two-photon and multiphoton excitation (TPE and MPE, respectively) using high-power, near-infrared femtosecond lasers. MPE is an effective technique whose capability relies on nonlinear interactions between short (100 fs) laser pulses at a high repetition frequency (80 MHz) and the sample, which leads to the simultaneous absorption of two or more photons within the excitation process.

Because of this nonlinear dependence on illumination intensity, fluorescence is expressed strictly in the vicinity of the focal plane, thus reducing out-of-focus effects, improving signal detection, and performing intrinsic optical sectioning.

Moreover, the use of longer wavelengths (with respect to single photon microscopy) couples a deeper penetration power with less overall phototoxicity, which makes this technique particularly useful in the presence of delicate targets such as biological samples.

Multiphoton (MP) uncaging for nitrophenyl-EGTA, which is one of the most popular caged Ca2+ compounds, has been shown to be scarcely efficient on the millisecond time scale, because of the low MP cross section of these chromophores. Also, 4,5-dimethoxy-nitrophenyl-EGTA, which is obtained from nitrophenyl-EGTA via the addition of methoxy substituents at positions 4 and 5, proved to be functional for MP uncaging, also on a submillisecond time scale. A compound that is based on a similar chromophore (a 2-nitrobenzyl system modified with methoxy functionalities at positions 4 and 5) but with lower uncaging yield, dimethoxy-nitrophen, was shown to be incapable of producing appreciable Ca2+ release on millisecond time scales. The near-UV, nπ* electronic transition of unmodified 2-nitrobenzyl-based caged compounds do not possess a sufficiently high TPE or MPE cross section to cause rapid (submillisecond) and extensive photolysis upon excitation in the near-infrared range of the laser powers used in the current experimental setups. However, it is of general interest to investigate the possibility of obtaining MP uncaging also under steady-state conditions, where dynamics is less important, whereas spatial localization of the uncaging process is crucial. Furthermore, a combination of MP cross section and photolysis yield is expected to control the effectiveness and speed of the uncaging.

A few compounds that function as caged protons capable of imposing rapid net acidification have been described previously. Among these is 2-hydroxyphenyl-1-(2-nitrophenyl)-ethyl phosphate. More recently, a new caged proton compound, 1-(2-nitrophenyl)-ethyl sulfate, has been characterized that promises to be able to achieve large pH jumps in solution.
For monitoring the local release of H+ in a cellular system but with a pulsed UV laser.

Materials and Methods

Materials. Fluorescently caged proton, 14 was used as a photolabile proton source. All chemicals were utilized without further purification. Milli-Q-grade water (Millipore Corp., Bedford, MA) was used in all solutions and in the experimental and cleaning steps. The compound 2-nitrobenzaldehyde (o-NBA) was obtained from Sigma—Aldrich and was recrystallized from ethanol before use.

Methods. The preparation of the calcium carbonate crystals primarily followed the method that was described by Kitamura24 and was based on the polymorphism between vaterite (spherical) and calcite (rhombohedral). The Na2CO3 solution used to prepare the nanocapsules was prepared by mixing Na2CO3 (anhydrous), MgCl2·6H2O, and CaCl2·2H2O in a 0.5 M NaCl solution were coated via deposition of the polyelectrolytes and a fluorescent dye. Polyelectrolytes, poly(styrene sulfonate sodium salt) (PSS, with a molecular weight of MW = 70 000 Da) and poly(allylamine hydrochloride) (PAH, MW = 15 000 Da), as well as the pH-sensitive fluorescent dye fluorescein isothiocyanate (FITC), were obtained from Aldrich (Milan, Italy). PAH was labeled with FITC following a labeling protocol that has been described in detail elsewhere.22,23 The removal of unbound FITC was performed by dialysis against Milli-Q-grade water or 0.5 M NaCl solution for at least one week by means of a dialysis membrane with a cutoff of 3.5 kDa (Spectrum Laboratory Products, Gardena, CA).

The chemicals used to prepare the calcium carbonate crystals (Na2CO3 (anhydrous), MgCl2·6H2O, and CaCl2·2H2O) were purchased from Sigma (Milan, Italy). All chemicals were utilized without further purification. Milli-Q-grade water (Millipore Corp., Bedford, MA) was used in all solutions and in the experimental and cleaning steps. The compound 2-nitrobenzaldehyde (o-NBA) was obtained from Sigma—Aldrich and was recrystallized from ethanol before use.

The nanocapsule preparation followed the layer-by-layer method that has been previously described in detail.22,26 These capsules were called nanocapsules in the following discussion, because of their nanostructured capsule walls. Despite the size, which can vary from tens of nanometers to hundreds of micrometers, the thickness of nanocapsules can be controlled (within the nanometer scale), as well as their permeability.22,26

In this paper, we report some preliminary results on the two-photon photolysis of o-NBA, which leads to the release of protons within fluorescent-labeled polyelectrolyte capsules. The coupling of TPE with FITC-tagged nanocapsules is a key step for monitoring the local release of H+ ions from o-NBA. To our knowledge, this is the first report of a multiphoton-induced decrease in pH using a photolabile compound. Previous studies showed the possibility of reducing the pH via the photolysis of 2-hydroxyphenyl-1-(2-nitro)-phenylethyl phosphate (NPE-caged phosphate) in a cellular system but with a pulsed UV laser.10

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NBA was dissolved in Milli-Q-grade pure water, and the pH was set to 7 through the addition of proper volumes of NaOH and HCl solutions. The concentration was adjusted to obtain an absorbance of 0.1 over a 1-cm optical path at 260 nm.

Finally, the solution was mixed with an aqueous suspension (pH 7) of FITC-labeled nanocapsules.

Image Acquisition and Analysis

A drop of sample was placed on a glass coverslip (nominal thickness of 0.17 mm) and imaged at 720 nm with a mode-locked Ti:sapphire infrared pulsed laser (model Tsunami 3960, Spectra Physics, Mountain View, CA) that was focused through a Nikon 1.3-NA 100× oil-immersion objective. The input power to the scanning head was ~20 mW (~7 mW on the focal plane) and a 535HQ (selective for fluorescein emission) was used as a barrier filter ahead of the photomultiplier tube (PMT) detectors.28

Within the 70 μm × 70 μm field of view previously imaged, a small region (13 μm × 13 μm) was chosen; this small area, which is described in the following, is called the uncaging spot. This area, which is in the direct neighborhood of the nanocapsules, was the focus point of the laser beam, and the region was scanned for ~1 min at high power (45–50 mW) and the laser acted on a neutral-density filter to induce uncaging of the protons. The power then was reduced to the commonly used 20 mW power that is used for scanning, and this region was imaged again. The nanocapsule that contained buffer solution without NBA served as a control, to reveal photobleaching effects or other experimental artifacts (such as x-y-displacement or z-defocusing) that may be due to our laser-scanning device. Imaging conditions and parameters were maintained constant for each set of measurements.

Images were analyzed by first reducing background noise, through the application of a median filter (3 pixels in size). Furthermore, a spatial offset parameter was calculated to shift one region to another and to obtain a more precise overlapping between them. This algorithm, which was developed within Matlab (The Mathworks, Natick, MA), calculates an average center displacement vector of the manual setting of the corresponding points in the two images (Figure 2). After this alignment procedure, the image recorded after the uncaging was subtracted from the image that was recorded before the uncaging, and data analysis was performed on the pixel intensity of this difference array. The number of pixels that correspond to each possible intensity level is counted: positive levels account for a decrease of fluorescence emission from the sample whereas negative levels account for an increase of it. Background pixels whose intensity level is zero in both of the images are also counted separately, to exclude them from further statistical calculations. An estimate of the average intensity for each data acquisition is calculated, according to the following relationship in which background pixels are excluded:

\[
\text{average value} = \frac{\sum_{s=1}^{256} \left( n^+ - n^- \right)}{\sum_{s=1}^{256} n^+ - \sum_{s=1}^{256} n^-}
\]

where \(n^+\) is the percentage of the pixels (in the difference array) whose intensity level is \(s\) and \(n^-\) is the percentage of the pixels (in the difference array) whose intensity level is \(-s\).

Results

The decrease in FITC intensity that is due to pH changes can be observed by comparing the TPE images before and after uncaging protons by photolysis for samples without NBA (Figure 3A) and more clearly for capsule suspensions that contain NBA in the buffer solution (Figure 4A). These results are supported by the image analysis presented in Figures 3B.

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intensity level, which is expected for H⁻ in the presence of NBA, most of the pixels have a positive value in the difference image, whereas, pixel intensities in the absence of NBA. Therefore, the latter effect can be correlated with the photolysis of the o-nitrobenzyl compound and the release of protons to the solution. The data sets reveal that the observed decrease in fluorescence emission when the sample while the uncaging process is underway. It is reasonable to expect that, while high-power laser scanning is performed, photoreleased protons partially leave the focal volume in the sample while the uncaging process is underway. It is reasonable to expect that, while high-power laser scanning is performed, photoreleased protons partially leave the focal volume in the surrounding solution. Note that the local decrease of pH, and, thus, that of fluorescence, could be larger than we were able to observe. So far, we have been able to evaluate changes in the overall fluorescence emission only after a 1-min two-photon photolysis has been completed, because we cannot image the sample while the uncaging process is underway. It is reasonable to expect that, while high-power laser scanning is performed, photoreleased protons partially leave the focal volume in the microsecond and millisecond time scale, because of diffusion processes.7,15

The results show that, when high-powered scanning is conducted without NBA in the solution, this procedure does not lead to a significant change in the image average intensity: the percentage of positive intensity pixels in the difference array is approximately the same as, although slightly smaller than, the percentage of negative intensity pixels (see Figure 3B). The fact that the percentage of pixels with a positive intensity value is always bigger than that of pixels with a negative intensity value is consistent with a certain intrinsic decrease of fluorescence in both cases (with or without NBA), because of photobleaching effects. This finding shows that photobleaching effects on fluorescein represent only a minor contribution to the observed decrease in fluorescence emission when the sample also contains NBA. Therefore, the latter effect can be correlated with the photolysis of the o-nitrobenzyl compound and the release of protons to the solution. The data sets reveal that the overall fluorescence decrease is 4—5 times stronger when NBA is present in the nanocapsules solution (see Figure 4B). In Table 1, the results of a typical image analysis are listed. Each row is related to the statistics that have been performed over the difference array of a single data acquisition. In the top portion of the table (the first eight rows of data), the table reports data from eight data sets that have been derived from separate uncaging experiments. The bottom portion of the table shows data sets derived from control experiments in the absence of NBA. * Value given in boldface type represents the average value of the set of data that is presented.

Figure 4. (A) Two-photon excitation images of FITC-doped poly-electrolyte nanocapsules ((PAH/PSS), 3rd and 5th layer FITC-labeled). Fluorescein emission was excited at a wavelength of 720 nm, in the presence of 2-nitrobenzaldehyde, before (upper image, right row) and after (lower image, right row) 2-nitrobenzaldehyde photolysis that was induced by high-energy scanning with multiphoton excitation at 720 nm. (B) Image analysis showing the percentage of the number of pixels (%) positive intensity level (decrease) and (O) negative intensity level (increase)) versus the number of intensity-level intervals, in the presence of NBA.

Table 1: Statistics Performed over the Difference Array, Listed on Each Row for Every Single Data Acquisition

<table>
<thead>
<tr>
<th>Sample</th>
<th>Positive Level (%)</th>
<th>Negative Level (%)</th>
<th>Average Intensity Difference (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With NBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sample 1</td>
<td>84</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>sample 2</td>
<td>84</td>
<td>15</td>
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<td>33</td>
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</tr>
<tr>
<td>sample 7</td>
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<td>23</td>
<td>15</td>
</tr>
<tr>
<td>sample 8</td>
<td>77</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Without NBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sample 1</td>
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<td>41</td>
<td>5</td>
</tr>
<tr>
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<td>55</td>
<td>42</td>
<td>3</td>
</tr>
<tr>
<td>sample 3</td>
<td>52</td>
<td>47</td>
<td>2</td>
</tr>
<tr>
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<td>50</td>
<td>0</td>
</tr>
<tr>
<td>sample 5</td>
<td>58</td>
<td>40</td>
<td>8</td>
</tr>
</tbody>
</table>

* The upper portion of the table refers to different data sets from separate uncaging experiments in the presence of 2-nitrobenzaldehyde (o-NBA) and, therefore, a pH decrease. The lower portion of the table shows data sets derived from control experiments in the absence of NBA. * Value given in boldface type represents the average value of the set of data that is presented.
Substantial improvements in the photolysis rate are expected to occur through the use of a photosensitive chromophore, for instance, the 4,5-dimethoxy derivative of 2-nitrobenzaldehyde. Although the addition of methoxy substituents is known to reduce the photochemical yield of the reaction, an enhancement of the two-photon cross section in the near-infrared range is expected to occur,9 which, hopefully, more than compensates for the decrease in efficiency.

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References and Notes