Rose Bengal Promoted Catalytic Amyloid-β Oxygenation via Sono-Activation

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Abstract Catalytic photo-oxygenation of amyloid-β is a leading therapeutic strategy for the treatment of Alzheimer disease. However, the limited tissue-permeability of light hampers its clinical application. We here report an alternative catalytic sono-oxygenation strategy to circumvent this problem. Amyloid-β aggregates were oxygenated using rose bengal as a sonosensitizer under ultrasound irradiation. Structure-activity relationships revealed that xanthene-derived catalysts containing halogen atoms furnished superior amyloid oxygenation activity.

Key words Alzheimer disease, amyloid-β, amyloid oxygenation, ultrasound, sonodynamic therapy, sonosensitizer

Introduction

With the average age of the population continuing to rise, the number of patients with dementia is increasing year by year and is expected to reach approximately 130 million worldwide by 2050. Alzheimer disease (AD) is one of the most common causes of dementia. AD is a progressive and chronic neurodegenerative disease that leads to memory impairment, cognitive decline, and loss of autonomy. Microscopic examination of AD patients’ brain tissue reveals the presence of two characteristic pathological lesions: neurofibrillary tangles and senile plaques. They are characterized as containing aggregates composed of tau protein and the amyloid-beta (Aβ) peptide, respectively. Aβ is thought to cause AD through the formation of its aggregate, which is called the amyloid hypothesis. As Aβ aggregates have been reported to induce neurodegeneration, targeting Aβ aggregates may afford treatment strategies for AD. In the pursuit of a novel treatment for AD, we and others reported catalysts capable of photo-oxygenating Aβ (Scheme 1a). Specifically, our catalysts furnished the cross-sheet-sensing switch enabling amyloid-selective photo-oxygenation under visible-near infrared light irradiation. The catalyzed photo-oxygenation of Aβ attenuated its aggregative propensity and toxicity. Furthermore, Aβ photo-oxygenation in vivo promoted clearance of Aβ from the AD-model mouse brain through enhanced microglial degradation of photo-oxygenated Aβ, suggesting therapeutic potential.

However, there is a potential drawback regarding the catalytic photo-oxygenation strategy; the poor biopermeability of light hampers clinical applications in humans. As the skin thickness is much thicker than that of a mouse, it is challenging to activate photo-oxygenation catalysts in the brain by photo-irradiation. Ultrasound is an attractive alternative energy source from the standpoint of medical applications. For example, sonodynamic therapy (SDT) is a novel therapeutic approach that enables cancer treatments deep inside the body, something that is not possible with photodynamic therapy (PDT). The superior permeability of ultrasound to light makes the activation of sensitizers in deeper tissues possible. Therefore, we hypothesized that amyloid oxygenation could be achieved in the human brain if a suitable catalyst, able to be activated by ultrasound, could be identified (Scheme 1b).
Result

As an initial study, we optimized ultrasound irradiation conditions using rose bengal as a sonosensitizer (Figure 1a). Among the parameters of ultrasound, irradiation intensity and time were examined. In recent SDT studies, the intensity has generally been within the range of 0.5–25 W/cm², and the irradiation time has been about 10 minutes, at most. The reaction conditions were optimized based on the oxygenation yield of aggregated Aβ (see Materials and Methods), which was calculated by MALDI-TOF MS analysis. DMSO was used as vehicle control. The main photo-oxygenation site of amyloid is histidine and methionine (Figure 1b).

First, we optimized the ultrasound intensity by fixing the irradiation time at 10 minutes (Figure 1c). The oxygenation yield increased according to the intensity, producing the highest oxygenation yield (12%; yield described in this study is an average value based on at least two independent experiments) at 5.0 W/cm². Next, the intensity was fixed at 5.0 W/cm² and the irradiation time was changed from 10 to 20 minutes (Figure 1d). 20-minute irradiation afforded a slightly higher oxygenation yield (14%) than 10-minute irradiation. We confirmed that rose bengal was stable under the photo-oxygenation conditions based on the comparison of the absorption spectra before and after the reaction. It was also found that rose bengal promoted oxygenation in 7% yield without ultrasound. This was likely due to photo-oxygenation by the background light of the fluorescent lights in the laboratory, despite that the reaction vessel was shaded during the reaction. However, as the effect of ultrasound was still apparent, the intensity of 5.0 W/cm² and the irradiation time of 20 minutes were employed as the optimal conditions.

Next, we applied the optimized ultrasound irradiation conditions to amyloid-selective photo-oxygenation catalysts 1–4 previously developed in our group (Figure 2). However, none of those catalysts showed any sonodynamic activity.

Based on these findings, we screened a further five xanthene derivatives (erythrosin B, phloxine B, eosin Y, fluorescein, and rhodamine B) under the sonooxygenation conditions (Figure 3). The oxygenation reaction proceeded only slightly when using the catalysts bearing halogen atoms (erythrosin B, phloxine B, and eosin Y promoted oxygenation in 7%, 3%, and 3% yield, respectively, without ultrasound). These results revealed that...
rose bengal has the highest activity among these xanthene derivatives.

![Figure 3](image)

**Figure 3** Screening of xanthene catalysts

Frequency: 901 kHz, Intensity: 5 W/cm², Irradiation time: 20 min, Duty ratio: 33% (ON: OFF = 5 ms : 10 ms), n = 2, means ± SE.

**Discussion**

Although the mechanism of sono-sensitization remains elusive, cavitation may cause the generation of active oxygen species. Cavitation is a unique physical phenomenon caused by bubbles that form during ultrasound irradiation. It has been reported that the bubbles undergo a series of steps of growth, resonance, and collapse, releasing enormous amounts of energy. As a result, various effects, such as mechanical and thermal effects, are also observed. It has been suggested that one such effect, a type of light emission called sonoluminescence (SL), is involved in the activation of sensitizers. In 1995, Matula reported the spectrum of multibubble sonoluminescence in 0.1 M NaCl solution. In this research, the emission peaks around 310 nm and 589 nm due to the hydroxy radical (OH·) and the sodium D-derived peak, respectively, were observed. Also, Beguin showed the involvement of SL in the excitation mechanism of rose bengal.

On the basis of these reports, we assume that SL was involved in the mechanism of Aβ oxygenation. The proposed mechanism is shown in Figure 4. First, the sensitizer is activated to the singlet excited state via intersystem crossing and decays to the ground state, providing reactive singlet oxygen. This species reacts with nearby Aβ to yield the oxygenated product.

**Figure 4** Proposed reaction mechanism

The oxygenation reaction proceeded only when using catalysts containing halogen atoms (Figure 3). This can be explained by the heavy atom effect brought about by the halogen atoms, which promoted the intersystem crossing. In addition, rose bengal has been reported to have an affinity for Aβ aggregates. These attributes of rose bengal may be responsible for the observed oxygenating activity. While oxygenation yield is still low, we previously found that the oxygenated Aβ works as an aggregation inhibitor of native Aβ. Due to this property, 10–15% oxygenation yield was enough to attenuate the toxicity of Aβ.

In summary, we worked on the development of amyloid sonooxygenation catalysts to overcome the shortcomings of conventional photo-oxygenation strategies. After various condition studies and screening, rose bengal was identified as a small-molecule catalyst capable of catalyzing sonooxygenation of amyloid. Further investigation of the catalyst structure and reaction conditions are currently underway.

**Materials and Methods**

**General**

Ultrasound irradiation experiments were performed using Sonitron GTS purchased from Nepa Gene for plane wave mode (Nepa Gene Co., Ltd., Chiba, Japan). MALDI-TOF MS was measured on a Shimadzu Biotech Axima-ToF2 TM spectrometer (Shimadzu, Co., Kyoto, Japan) using α-cyano-4-hydroxy cinnamic acid (Sigma-Aldrich, Inc., St. Louis, MO, U. S. A.) as a matrix. We acquired and averaged many (200) single-shot spectra from several positions within an identical sample spot to obtain representative sample data. Absorbance measurement was performed using a Shimadzu UV-1800 spectrometer with a rectangular quartz cell (5 mm pathlength). The temperature of the sample solution was measured using a digital thermometer (model SK-1260) purchased from skSATO (SATO KEIRYOKI MFG. Co., Ltd., Tokyo, Japan). Water was purified in advance using a Millipore Milli-Q water purification system (Merck KGa, Co., Darmstadt, Germany) to obtain filtered deionized water and distilled water.

**Materials**

The isopeptide, Aβ1-42 was purchased from Peptide Institute, Inc. (Osaka, Japan). Lysyl endopeptidase® (Lys-C), mass spectrometry grade was purchased from FUJIFILM Wako Pure...
Preparation of Aβ1-42 aggregates

Aβ1-42 aggregates were prepared from the 26-O-acyl isopeptide in situ as described in the previous report[16]. A stock solution of Aβ1-42 isopeptides (200 µM in 1% aqueous trifluoroacetic acid) was diluted with 0.1 M phosphate buffer to a final peptide concentration of 20 µM (pH 7.4). The solution was incubated at 37 °C for 3 h to acquire an aggregated Aβ1-42 sample.

Sono-oxygenation of aggregated Aβ1-42

To the Aβ1-42 solution (25 µL) prepared as above, 0.5 µL of a catalyst solution (500 µM in DMDO) was added (the final concentration of catalyst: 10 µM). The mixture was then subjected to ultrasound irradiation at room temperature under appropriate conditions. During the ultrasound irradiation, the apparatus was shielded to prevent undesirable photo-oxygenation reactions. Reaction samples without ultrasound irradiation were also prepared as controls. The sample was digested with Lys-C overnight. Prior to the MS analysis, the aliquot of reaction samples was desalted with ZipTip U-C18 (Millipore Corporation). The sample was analyzed using MALDI TOF MS. The fragment containing histidine residues (HDSGVYEHQHK) was detected and used to calculate yields, while the fragment containing methionine residue (GAILGMLGGRQVA) was not detected. The yield of oxygenation was expressed as the intensity ratio of oxygenation (‰) = (sum of MS intensities of n[O] adducts) / (sum of MS peak intensities for remaining starting material and n[O] adducts) × 100.

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Conflict of Interest

There are no conflicts to declare.

References and Notes

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