

# Stabilizing and Prolonging the Antioxidant Activity of Catalase Using Nondegradable Nanoparticles

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(Received June 23, 2023; accepted October 2, 2023)

**Keywords:** nanoparticle, catalase, stabilization of enzymes, mesh structure, antioxidative stress

Inflammation induced by oxidative stress causes further stress through positive feedback and may lead to various diseases. Antioxidant enzymes such as catalase can be useful therapeutic agents; however, their applicability is currently limited by their low stability. The aim of this study was to increase the stability of catalase by encapsulating this enzyme in nondegradable nanoparticles based on polyethylene glycol. Nanoparticle formation was confirmed using nanotracking analysis and transmission electron microscopy. The mesh structure of the nanoparticles allowed the passage of only very small molecules such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The catalase encapsulated in the nanoparticles was stabilized because of isolation from destabilizing external factors such as proteases and was capable of decomposing  $\text{H}_2\text{O}_2$  as determined by measuring the catalase activity. Upon introduction into HeLa cells, the nanoparticles protected the cells from oxidative stress caused by  $\text{H}_2\text{O}_2$ , which was confirmed by analyzing microscopy images and determining the number of live cells by cell staining. In the future, this method can ensure the continuous antioxidant activity of catalase in inflammatory cells and allow the retention of activities of other types of enzyme for extended periods within the cells, and also expected to be used as an intracellular sensor for monitoring enzyme function within cells.

## 1. Introduction

Oxidation reactions are involved in various physiological processes such as adenosine triphosphate (ATP) production, which is necessary for life. During ATP production, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) generated in the ATP cycle causes oxidative stress. The produced  $\text{H}_2\text{O}_2$  is typically decomposed by an antioxidant molecule such as catalase in cells. If this decomposition is inhibited, hydroxyl radicals remain in the cells, and their strong oxidizing activities will oxidize lipids, damage intracellular DNA, and lead to tissue inflammation. Inflammation causes

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<https://doi.org/10.18494/SAM4557>

further stress in a positive feedback manner, which may lead to various diseases such as Alzheimer's disease, Parkinson's disease, and depression.<sup>(1–5)</sup> Therefore, reducing oxidative stress is useful for preventing and treating various chronic diseases, and several methods have been developed.<sup>(6–8)</sup>

The enzyme catalase exhibits antioxidant effects against  $H_2O_2$  and shows promise as a therapeutic agent for chronic diseases. However, enzyme isolation and purification processes are expensive; moreover, enzymes exhibit low stability, and impurities must be removed before use.<sup>(9)</sup> Furthermore, enzymes are decomposed by various proteases and downregulated by immune responses, and their introduction into cells is inefficient.<sup>(10)</sup> To overcome these limitations, the immobilization of enzymes on solid supports such as nanoparticles has been extensively studied to improve enzyme stability and extend their functional life.<sup>(11–14)</sup> Nanoparticles have been used to encapsulate catalase; however, they release catalase upon light exposure or via leakage over time.<sup>(10–11)</sup> Therefore, when the released catalase molecules are decomposed by proteases, they lose their activity.

In previous studies, we regulated enzyme function using nanoparticle gels prepared from photoresponsive hydrophilic cross-linkers and used these gels to encapsulate various enzymes.<sup>(12,13)</sup> In nanoparticle gels, the cross-linker forms a mesh structure (approximately 14 nm) that hinders the movement of macromolecules such as enzymes.<sup>(14)</sup> In contrast, water can freely access the interior of nanoparticles by passing through gaps in the mesh structure. Therefore, nanoparticles can retain an enzyme under hydrophilic conditions without modification. These nanoparticles decompose upon light exposure and release the enzyme, thus allowing the enzyme to perform its activity at a specific location and time. These studies indicate that very small molecules such as water can access the enzyme in nanoparticles.<sup>(15,16)</sup> In this study, we exploited such characteristics to stabilize catalase and protect it from external proteases, and these nanoparticles would be applied as an intracellular sensor for monitoring enzyme function within cells.

We hypothesized that  $H_2O_2$  (molecular weight: 34.0) can easily access enzymes contained in nanoparticles by passing through the mesh structure of the nanoparticle gel, similar to water. Furthermore, by using a smaller cross-linking agent (2 kDa) and adding a small linker such as acrylamide, enzyme leakage from the nanoparticles or the effects of external proteins on the enzyme may be prevented, allowing the enzyme to remain active for longer durations. Additionally, catalase might decompose  $H_2O_2$  without being released from the nanoparticles. To evaluate these assumptions, we designed such nanoparticles and evaluated their enzymatic activity.

In the future this method could not only ensure the continued antioxidant activity of catalase in inflammatory cells, but also allow other enzymes to work in the cell. Changing the amount of target biomolecule by the encapsulated enzymes allows us to monitor enzyme function. Thus, it is expected to be applied to intracellular sensors and probes for measuring and controlling intracellular enzyme functions.

## 2. Materials and Methods

### 2.1 Materials

The uncleavable linker ATM-35E (X-linker) was purchased from SHIN-NAKAMURA CHEMICAL Co., Ltd. (Wakayama, Japan).  $H_2O_2$ , *N,N,N',N'*-tetramethylethylenediamine (TEMED), acrylamide, acrylic acid chloride, and ammonium persulfate were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). DAB-AM-4, polypropylenimine tetramine dendrimer, generation 1 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was purified with a Milli-Q system (Millipore; Billerica, MA, USA).

### 2.2 Preparation of cationic linker

The cationic linker (DAB-Ac) was synthesized as previously described.<sup>(17)</sup> Briefly, DAB-AM-4, polypropylenimine tetramine dendrimer, generation 1, and acrylic acid chloride were reacted with Kunishima's reagent.<sup>(18)</sup>

### 2.3 Preparation of nanoparticles

First, 200  $\mu$ L of 25% X-linker with 0.5 M acrylamide, 100  $\mu$ L of DAB-Ac, and 100  $\mu$ L of 0.5 U/mL solution of catalase were added to a microtube and shaken for 5 s. Next, 50  $\mu$ L of 0.1 mol/L ammonium persulfate and 50  $\mu$ L of 0.1 mol/L TEMED were added to the mixture to induce radical polymerization at room temperature (15–25 °C). The mixture was vortexed for 20 min to prepare catalase-containing nanoparticles. The nanoparticles were washed with water and collected using a Vivaspin 6 MWCO 30000 polyethersulfone centrifugal concentrator filtration system (Sartorius, Göttingen, Germany). The samples were passed through the concentrators twice at  $3000 \times g$  for 20 min at 4 °C.

### 2.4 Measurement of catalase activity in vitro

Catalase activity was determined in vitro using the AAT Bioquest® Amplite™ Fluorimetric Catalase Assay Kit (AAT Bioquest, Newport Beach, CA, USA) in accordance with the manufacturer's protocol. Briefly, the washed nanoparticles were mixed with  $H_2O_2$  and incubated for 30 min. A fluorescent substrate was added, and the fluorescence intensity (Ex/Em = 540/590 nm) was measured using a plate reader (SH-9000Lab; Hitachi High-Tech, Tokyo, Japan) to measure antioxidant activity. The fluorescence intensity was converted to the amount of active catalase using a calibration curve.

## **2.5 Measurement of nanoparticle size distribution, concentration, and zeta potential through nanoparticle tracking analysis**

After washing with water as described above, the nanoparticle solutions were diluted 200-fold with 0.1 mol/L sodium chloride and evaluated under a nanoparticle-tracking microscope (ZetaView, MicrotracBEL; Analytik, Cambridge, UK). The laser light scattered by the nanoparticles was recorded using a camera attached to the ZetaView microscope. The diffusion coefficient and electrophoretic mobility were determined computationally on the basis of the movement of scattered light. The nanoparticle size was calculated from the diffusion coefficient using the Stokes–Einstein equation, nanoparticle surface charge was calculated from the electrophoretic mobility using the Smoluchowski coagulation equation, and nanoparticle concentration was calculated from the total number of particles in the camera field of view with known capacitance using ZetaView 8.04.02 SP1 software.

## **2.6 Nanoparticle size determination using transmission electron microscopy (TEM)**

The nanoparticles were negatively stained with 2% uranyl acetate, and their sizes were examined using a transmission electron microscope (H-7600, Hitachi High-Tech; Tokyo, Japan).

## **2.7 Stability test of catalase-containing nanoparticles with external proteases**

The washed nanoparticles (200  $\mu$ L) containing catalase (final 0.1 U/mL) or free catalase (final 0.1 U/mL) were stored in 200  $\mu$ L of 10 mg/mL trypsin (30 USP units/mg; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 4 °C. The catalase activity of the samples was measured as described above on days 0, 7, and 14.

## **2.8 Continuous exposure test against nanoparticles**

Washed nanoparticles containing catalase (final 0.1 U/mL) or free catalase (final 0.1 U/mL) were mixed with an excess amount (1 mL) of 0.3% H<sub>2</sub>O<sub>2</sub> in a vial and incubated at 4 °C for 2 days. The nanoparticles and free enzyme were then washed with water and collected by ultracentrifugation with a Vivaspin 6 MWCO 30,000 (NP) / 3,000 (free), as described above, to remove the residual H<sub>2</sub>O<sub>2</sub>. The nanoparticles were tested for catalase activity by adding fresh H<sub>2</sub>O<sub>2</sub>, as described above.

## **2.9 Introduction of nanoparticles into cells**

HeLa cells were cultured until confluence (30  $\times$  10<sup>6</sup> cells in flask), diluted 10-fold, and then incubated at 37 °C for subculture. On the day before the assay, 200  $\mu$ L of the 10-fold-diluted confluent HeLa cells in DMEM with serum and 2.0 mL of DMEM with serum were seeded at 37 °C in a 35 mm glass-bottom dish (Matsunami Glass Ind. Ltd., Osaka, Japan). After overnight incubation, the dish was washed with DMEM without serum, and then 2.0 mL of DMEM was

added. A 50  $\mu\text{L}$  aliquot of the nanoparticle solution was added dropwise into the dish, followed by incubation at 37  $^{\circ}\text{C}$  for 20 min. The cells were then washed with 2.0 mL of PBS and supplemented with 2.0 mL of DMEM.

### 2.10 Toxicity test

After overnight incubation, the cells in a 96-well plate were washed with DMEM without serum and supplemented with 180  $\mu\text{L}$  of DMEM. A 20  $\mu\text{L}$  aliquot of the nanoparticle solution was added dropwise into the dish followed by incubation at 37  $^{\circ}\text{C}$  for 20 min. The cells were then washed with 200  $\mu\text{L}$  of PBS. Finally, 180  $\mu\text{L}$  of DMEM and 20  $\mu\text{L}$  of cell counting kit-8 (CCK8) solution (living cell staining agent: Dojindo Laboratories, Kumamoto, Japan) were added. After 20 min of incubation, cell death was evaluated by measuring the absorbance of the samples at 450 nm using a plate reader.

### 2.11 Effect of oxidative stress on cells

After overnight incubation, the cells in a 96-well plate were washed with DMEM without serum, and then 180  $\mu\text{L}$  of DMEM was added. A 20  $\mu\text{L}$  aliquot of the nanoparticle solution was added dropwise into the dish, followed by incubation at 37  $^{\circ}\text{C}$  for 20 min. The cells were washed with 200  $\mu\text{L}$  of PBS. Finally, 180  $\mu\text{L}$  of DMEM and 20  $\mu\text{L}$  of 0.3%  $\text{H}_2\text{O}_2$  were added. After 30 min of incubation, the cells were washed with 200  $\mu\text{L}$  of PBS to remove the  $\text{H}_2\text{O}_2$ , and then 200  $\mu\text{L}$  of DMEM was added. The cells were observed under an optical microscope (IX71; Olympus Corporation, Tokyo, Japan).

### 2.12 Measurement of cytotoxic activity induced by oxidative stress

On the day before the assay, 200  $\mu\text{L}$  of 100-fold-diluted confluent HeLa cells in DMEM with serum was seeded at 37  $^{\circ}\text{C}$  in a 96-well plate. After overnight incubation, the cells were washed with DMEM without serum, and then 180  $\mu\text{L}$  of DMEM was added. A 20  $\mu\text{L}$  aliquot of the nanoparticle solution was added dropwise into the dish and allowed to stand for 20 min at 37  $^{\circ}\text{C}$ . The cells were washed with 200  $\mu\text{L}$  of PBS, and then 180  $\mu\text{L}$  of DMEM was added. After introducing the nanoparticles into the cells, 0.3%  $\text{H}_2\text{O}_2$  (final concentration 0.03%) was added and incubated overnight to induce oxidative stress. Subsequently, the cells were washed with 200  $\mu\text{L}$  of PBS and supplemented with 180  $\mu\text{L}$  of DMEM and 20  $\mu\text{L}$  of CCK8 solution. After 20 min of incubation, the absorbance of the samples at 450 nm was measured using a plate reader.

### 2.13 Antioxidant stress test in cells

After introducing the nanoparticles containing catalase into the cells,  $\text{H}_2\text{O}_2$  solution was added (final concentration 0.03%), and the cells were incubated at 37  $^{\circ}\text{C}$  for 30 min to induce oxidative stress. After washing with PBS and removing the  $\text{H}_2\text{O}_2$ , the cells were stained with CCK8 solution, and the intensity of staining was evaluated using a plate reader. On the basis of the absorbance intensity (450 nm), the rate of cell death was evaluated using a calibration curve.

### 3. Results and Discussion

Nanoparticles were prepared using radical polymerization of the X-linker and acrylamide with catalase. To evaluate whether the substrate  $H_2O_2$  can access catalase by passing through the mesh structure and/or if catalase activity decreased because of damage or alteration caused by the radical polymerization reaction, we examined whether nanoparticulation reduced the catalase activity.

The nanoparticles containing catalase were washed with water and passed through a centrifugal filter to remove unencapsulated free catalase. Then, a catalase assay was performed with the remaining nanoparticles to examine their antioxidant activity (Fig. 1). Catalase in the nanoparticles showed an antioxidant activity similar to that of free catalase and could react with  $H_2O_2$  within the nanoparticle gel structure. This finding suggests that, under this nanoparticle condition (before polymerization of 0.04 or 0.1 U/mL catalase), the activity of catalase was saturated. Next, we confirmed whether catalase was active in the nanoparticles.

The physical properties of the nanoparticles were evaluated by nanoparticle tracking analysis (NTA) and TEM. The NTA results revealed that the size of the nanoparticles varied considerably, being approximately 150 nm in most cases, and they had a weak positive charge (less than 20 mV) [Figs. 2(a) and 2(b)]. The TEM results revealed the existence of multiple nanoparticles, some of them being spherical or collapsed, as shown in Fig. 2(c). These results suggest that catalase was encapsulated in the nanoparticles and exhibited antioxidant activity. On the basis of these properties, the nanoparticles were predicted to enter the cells by endocytosis, as previously reported.<sup>(19)</sup>

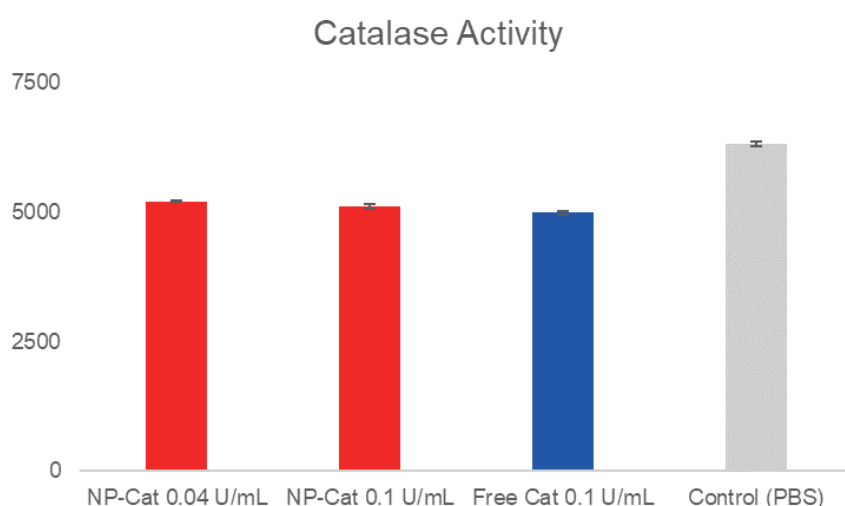


Fig. 1. (Color online) Catalase activity of nanoparticles. (Lower bars indicate stronger activity.) Catalase encapsulated in the nanoparticles (NP-Cat) showed an antioxidant activity similar to that of free catalase, suggesting that catalase can react with  $H_2O_2$  (concentration 0.03%) within the nanoparticle gel structure.



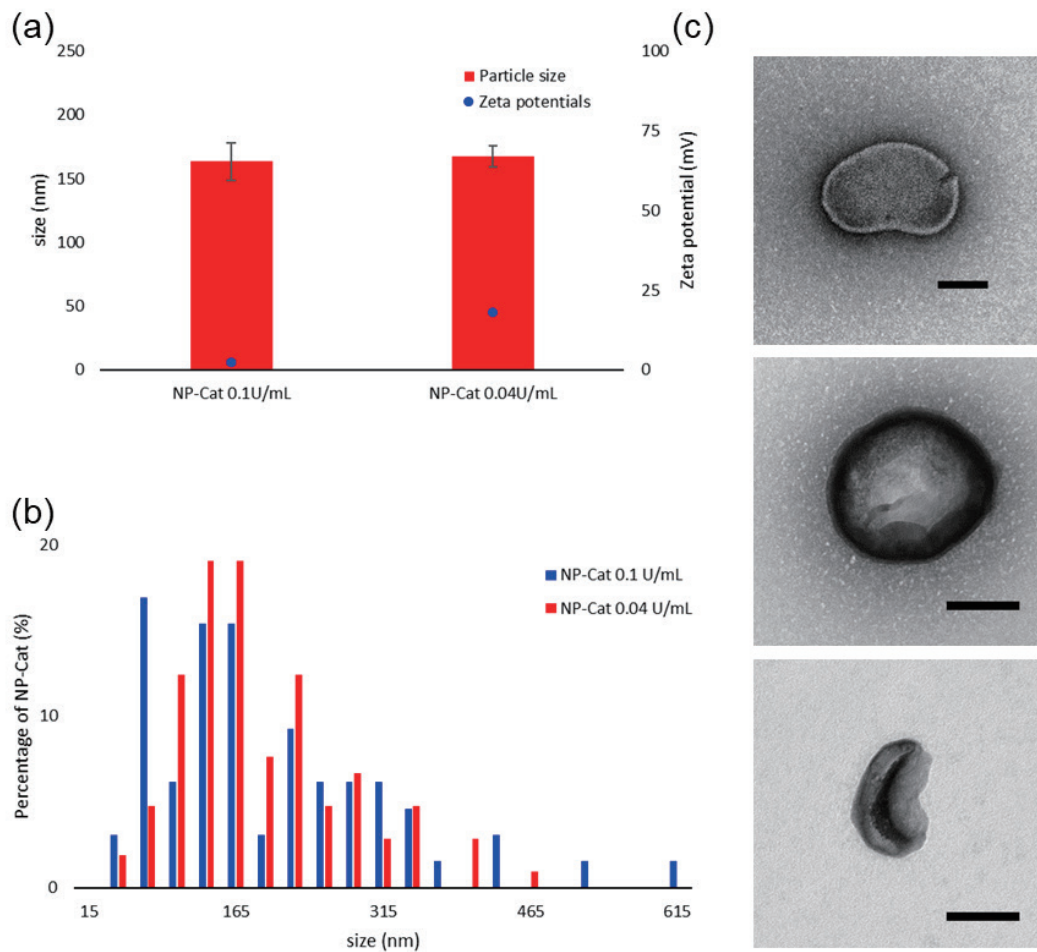


Fig. 2. (Color online) Physical properties of nanoparticles. Results of NTA (a, b) and TEM (c) of nanoparticles. a: NTA showed that NP-Cat has a weak positive charge (less than 20mV). Additionally, the structure (such as spherical and collapsed) and size (mostly 150 nm diameter regardless of the amount of encapsulated catalases) of the nanoparticles varied considerably. b: Graph showing a wide size distribution of NP-Cat. c: TEM images of negatively stained NP-Cat showing varying structures and sizes of nanoparticles; black bar, 100 nm.

In our previous study, an enzyme (protein) was isolated from the external environment with the gel mesh structure using nanoparticles prepared from a photoresponsive cross-linker (M/W m.5k).<sup>(12)</sup> The encapsulated enzyme retained its activity for a few days and could be reactivated by an external signal. In this study, nanoparticles were prepared from a nondegradable cross-linker with a lower molecular weight (2 kDa), and the enzyme (catalase) was isolated from the external environment using a mesh structure denser than that reported previously. Therefore, the enzyme was expected to be stable for a longer duration and was protected from proteases such as trypsin. To test this hypothesis, we evaluated how long the enzyme was stably retained in the nanoparticles in the presence of proteases. Catalase-encapsulating nanoparticles were prepared and stored at 4 °C for 2 weeks with a protease: trypsin. The activity of catalase was measured

and compared with that of free catalase. As shown in Fig. 3, free catalase showed a significant decrease in activity after 1 week of refrigerated storage and almost lost its activity after 2 weeks. This might be because of catalase degradation by trypsin. In contrast, nanoparticulate catalase was stably stored in the refrigerator for more than 2 weeks. In our previous study, the maximum reported enzyme storage duration in nanoparticle gels was 4 days,<sup>(12)</sup> however, in this study, the duration was extended to 2 weeks by eliminating photoreactivity and using a denser gel mesh structure. Moreover, it was protected from degradation by external enzymes.

The molecular size of  $H_2O_2$  is similar to that of water. Thus, we predicted that  $H_2O_2$  could pass through the extremely dense gel mesh structure and that catalase would exhibit the antioxidant activity within the nanoparticles. However, the continuous enzyme activity may decrease over time. Therefore, we investigated whether nanoparticles containing catalase exhibited oxidative stress-induced decomposition “continuously” for a longer duration.

To evaluate this effect, nanoparticles containing catalase and free catalase were stored at 4 °C for 2 days in the presence of excess  $H_2O_2$ . The nanoparticles were repeatedly washed with water using a centrifugal filter to remove the remaining  $H_2O_2$ . Fresh  $H_2O_2$  was added to the nanoparticles and free catalase, and antioxidant activity was measured. The results showed that the antioxidant activity of free catalase disappeared after 2 days, likely because of decomposition; however, catalase encapsulated in the nanoparticles retained its activity even after 2 days in the presence of  $H_2O_2$  (Fig. 4). Thus, the nanoparticles can be stored after preparation and can continue to decompose  $H_2O_2$  for at least 2 days. Furthermore, to test for cytotoxicity, the nanoparticle solution was tested on HeLa cells. In the cytotoxicity assays, exposure to the nanoparticles did not cause significant cell death compared with that in the control (Fig. S1).

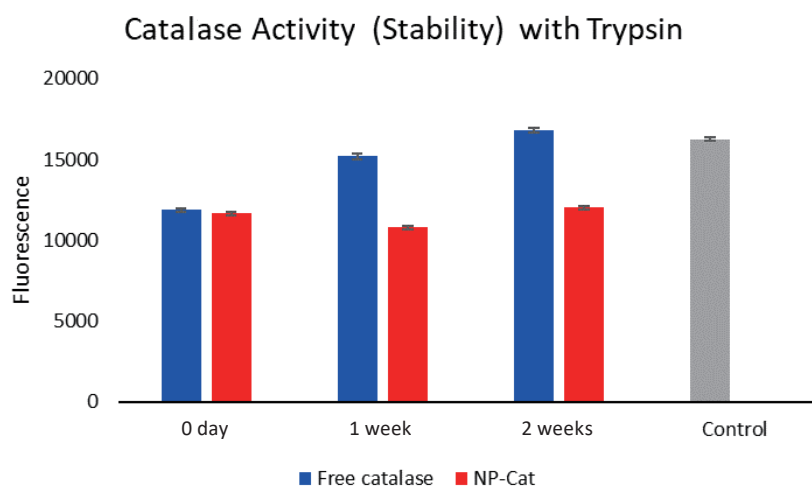


Fig. 3. (Color online) Stability of catalase activity in nanoparticles with trypsin. Catalase activity ( $H_2O_2$  degradation activity) under each condition is shown. ( $H_2O_2$  concentration 0.03%) There were no differences on day 0. The activity of free catalase with trypsin, decreased at 2 weeks; however, catalase activity was maintained using NP-Cat at 2 weeks in the presence of trypsin. Blue bar: free catalase; red bar: NP-Cat; gray bar: control (PBS with trypsin).



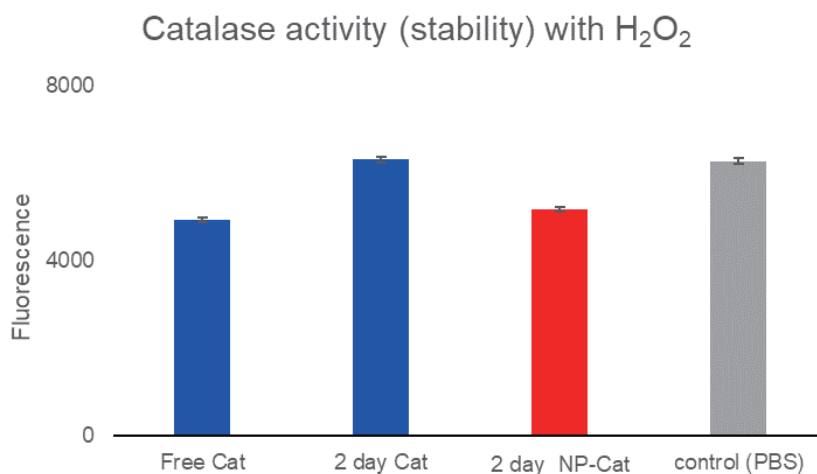


Fig. 4. (Color online) Continuous  $\text{H}_2\text{O}_2$  (concentration 0.03%) exposure test for the activity of catalase encapsulated in nanoparticles. Continuous  $\text{H}_2\text{O}_2$  exposure decreased catalase activity in 2 days. Catalase activity was maintained using NP-Cat even after 2 days in the presence of  $\text{H}_2\text{O}_2$  (concentration 0.03%). Blue bar: free catalase; red bar: NP-Cat; gray bar: control (PBS).

Next, the intracellular antioxidant activity of the nanoparticles containing catalase was examined. After 20 min of introducing the nanoparticles into HeLa cells via endocytosis, nonintroduced nanoparticles were washed out and lethal oxidative stress was induced by adding  $\text{H}_2\text{O}_2$  for 30 min.<sup>(20)</sup> After washing the cells with PBS to remove  $\text{H}_2\text{O}_2$ , changes in the cells were observed using an optical microscope [Figs. 5(a) and 5(b)]. In the absence of nanoparticles, oxidative stress caused significant cell death [Fig. 5(b)]. Upon nanoparticle addition, this cell death was not observed despite the addition of  $\text{H}_2\text{O}_2$  [Fig. 5(a)]. This result shows that nanoparticles containing catalase introduced into cells functioned within the cells, decomposed  $\text{H}_2\text{O}_2$  that had invaded the cells, and exhibited antioxidant stress activity to suppress changes in the cells.

We further quantitatively evaluated the effect of nanoparticles on cells. After introducing the nanoparticles, the cells were exposed to  $\text{H}_2\text{O}_2$  for 15 min to induce oxidative stress, washed with PBS to remove  $\text{H}_2\text{O}_2$ , and stained with CCK8 solution. The absorbance of CCK8 solution was measured. In the absence of nanoparticles, approximately 20% of cells were affected compared with that in the control; however, the nanoparticles mitigated the damage to the cells [Fig. 5(c)]. These results suggest that this nanoparticle-encapsulated catalase reduces oxidative stress in the cells through catalase activities. Also, we supposed that the nanoparticles that control enzymatic activity in cells could work as intracellular sensors for measuring enzymatic functions in cells.

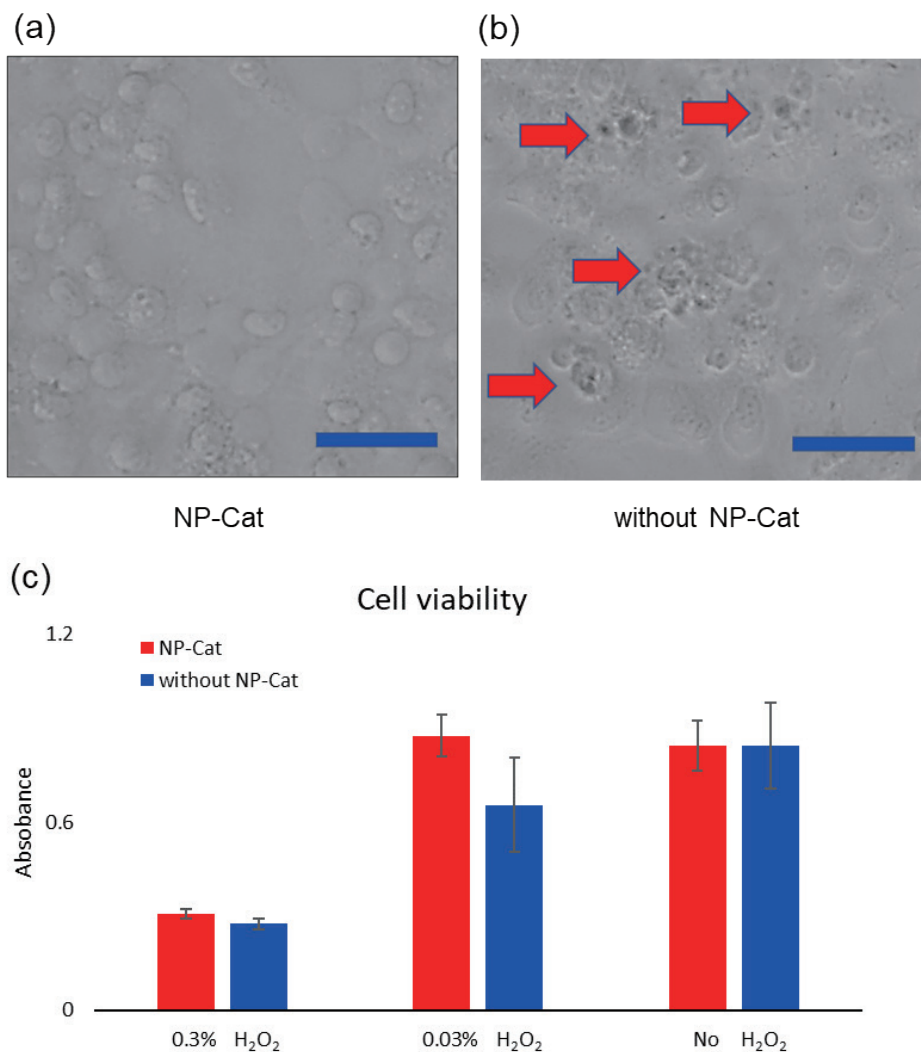


Fig. 5. (Color online) Antistress test in cells. (a, b) HeLa cells with NP-Cat (a) or without NP-Cat (b) 30 min after being exposed to 0.03% H<sub>2</sub>O<sub>2</sub>. NP-Cat prevented oxidative stress. Blue bar: 50  $\mu$ m. (c) Cell viability was measured by staining with CCK8 solution. The oxidative stress due to 0.03% H<sub>2</sub>O<sub>2</sub> was mitigated by NP-Cat.

#### 4. Conclusions

We prepared nondegradable nanoparticles with a dense gel mesh structure and used them to stably retain catalase in an active state. Catalase in the nanoparticles remained stable for 2 weeks in the presence of a protease (trypsin) and retained its activity after exposure to excess H<sub>2</sub>O<sub>2</sub> for 2 days. The introduction of these nanoparticles into the cells reduced oxidative stress caused by H<sub>2</sub>O<sub>2</sub> and suppressed cell death. The mesh structure constituting the nanoparticle gel inhibited the movement of catalase but not that of H<sub>2</sub>O<sub>2</sub>. By designing nanoparticles with a mesh structure that matches the size of the substrate compounds and encapsulates the enzyme, the enzyme

would function more stably and maintain its activity for a longer duration. Since such nanoparticles can control enzymatic activity in cells, the development of intracellular sensors for monitoring enzymatic functions in cells can be expected.

### Acknowledgments

This work was supported by JSPS KAKENHI (Grant Number 21K06497) and the JSPS Core-to-Core Program, A. Advanced Research Networks.

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