

Elucidation of the Mechanism of Near-Infrared Light-Induced Cell Death and Method Establishment for Measuring Therapeutic Effects

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Near-infrared photoimmunotherapy (NIR-PIT) is a new cancer treatment that involves the application of a near-infrared light probe (IR700) to a specific antibody that binds to antigens on the surface of cancer cells. However, despite its impressive efficacy and innovation, the detailed mechanism of cell death has remained unclear. From the viewpoints of analytical chemistry, condensed matter chemistry, and optics, it has been clarified that NIR-PIT cell death is the world's first photochemical-induced cell death, and the cell death can be measured and quantified by IR700 near-infrared fluorescence spectroscopy at 700 nm, thus establishing the basis for a therapeutic biomarker. Due to the novelty of this cell death, this therapy was recognized as a new cancer modality and was implemented clinically in Japan ahead of the rest of the world.



Introduction

Near-Infrared photoimmunotherapy (NIR-PIT) is a next-generation cancer-targeting therapy in which antibodies specific to antigens on the surface of cancer cells are attached to probes that react to near-infrared light and are then locally irradiated with near-infrared light (Figure 1)^[1]. This photo-targeted therapy is a futuristic, and multidisciplinary treatment method in which probes (chemistry) and antibodies (pharmacology and biology) are conjugated under optimal conditions (conjugation chemistry), and near-infrared light (optics and physics) is added (Figure 1). Irradiation with near-infrared light at 690 nm, the peak absorption of the probe, immediately expands the cancer cells and induces cell death. It is currently undergoing international Phase III trials (LUZERA-301)^[2,3] and was approved by PMDA in Japan in September 2020 for the treatment of recurrent and previously treated stage IV head and neck cancer^[4]. It is now being recognized as

the fifth cancer treatment after surgery, chemotherapy, radiotherapy, and cancer immunotherapy. So far, various application studies of NIR-PIT therapy have been conducted all over the world, and further development of this therapy is expected from here. Although this therapy has attracted attention for its innovation, the detailed mechanism of cell death in NIR-PIT had not been fully elucidated at the time of the introduction of Phase III trials. This was a barrier to its approval and implementation. In this review, we will provide an overview of NIR-PIT, which is attracting attention as a “fifth cancer therapy”, its cell death mechanism, and the prediction of its efficacy by near-infrared spectroscopic imaging derived from its elucidation.

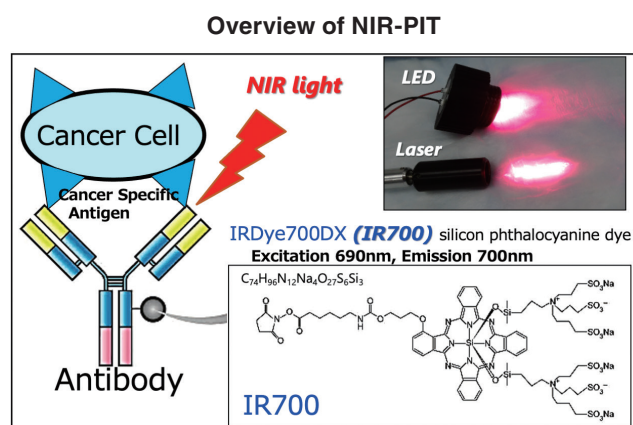


Figure 1 Overview of NIR-PIT
 NIR-PIT is a new therapeutic method in which an antibody-IR700 complex, in which IR700DX, a light-absorbing substance, is added to the antibody, binds to cancer-specific antigens that are highly expressed on the surface of target cancer cells, and is then exposed to NIR light.

About NIR-PIT

There are four known treatment methods for cancer: (1) surgery (surgical and endoscopic resection), (2) chemotherapy (anti-cancer drugs), (3) radiotherapy, and (4) cancer immunotherapy. These existing cancer treatments cause injury not only to cancer cells but also to surrounding normal tissues and organs. In anticancer drug therapy, chemical substances that cause damage to both normal cells and cancer cells are administered, but since the dosage is based on an extremely narrow dosage design that allows normal cells to recover earlier than cancer cells, the dosage is strictly determined, and if an overdose occurs, not only the cancer but also the individual may die. In addition, surgery requires the resection of normal tissues surrounding the cancer as margins, and radiation therapy inevitably causes injury to normal cells within and through the irradiation area. Therefore, it would be ideal if there were a cancer-targeted therapy that could superspecifically damage only cancer cells in the body without damaging normal cells and tissues.

Based on the above idea, Dr. Hisataka Kobayashi and his colleagues at the National Cancer Institute (NCI) in USA announced the development of Near-Infrared Photoimmunotherapy (NIR-PIT) in 2011^[1]. NIR-PIT is a photo-targeted therapy that combines near-infrared light and antibody photo-probe adducts, in which IR700, a light-absorbing probe, is covalently attached to antibodies that bind to cancer-specific antigens. NIR-PIT is super-selective because it is doubly targeted by NIR-light and antibodies (Figure 1).

NIR-light is used in TV remote controls, CD players, etc., and is a safe light that has almost no adverse effects when irradiated to the human body. IR700 is a probe that absorbs

light in the near-infrared region of 690 nm and emits fluorescence at 700 nm, and was originally developed by LICOR as a dye for microscopic observation in biology. Antibodies are biomaterials that have been actively used in medicine, biology, and engineering in recent years, and are among the existing biomolecules that can bind specifically and stably to molecules on the surface of cell membranes. Among the existing biomolecules, antibodies can bind specifically and stably to cell surface molecules^[4]. As described above, NIR-PIT is a multidisciplinary therapy that combines biology, pharmacology, chemistry, optics, engineering, and physics.

Mechanisms of cell death in NIR-PIT

It has been proposed that oxidative stress is the main mechanism of cell death using photosensitive substances^[5]. This pathway is often considered to be one in which the energy difference between photoexcitation and photoemission of a photosensitive substance acts on mitochondria and other organs, resulting in cell injury by oxidative stress agents such as singlet oxygen and free radicals, leading to cell apoptosis. The detailed mechanism of cell death in NIR-PIT has been unknown for a long time, which has been a barrier to its clinical implementation. Since the cell death reaction in NIR-PIT proceeded even after the cell function was stopped at 4°C, and the inhibition of cell death was not sufficiently effective even when oxidative stress inhibitors (free radical scavengers) were added, it was inferred that the oxidative stress was not the main mechanism for NIR-PIT. Therefore, it is inferred that the mechanism of NIR-PIT is not based on oxidative stress as in conventional photodynamic therapy (PDT).^[5]

NIR-PIT has a very different therapeutic mechanism compared to conventional cancer cell death. When the antibody-IR700 conjugate bound to the cell surface is irradiated with near-infrared light (690 nm), the cell surface is rapidly injured, the cell morphology changes, and then the cell bursts, leaking the cytoplasmic contents and causing cell death within a few minutes. Thus, the mechanism is necrosis (rapid destruction of cells) rather than apoptosis, which is the conventional slow death of cancer cells. Since near-infrared light irradiation itself is harmless to cells, it can only damage the target cells to which the antibody-IR700 conjugate binds. In fact, when cells with and without antibody-IR700 conjugates were co-cultured and irradiated with NIR light at the same time, cell death was observed only in the cells with antibody-IR700 conjugates, while the neighboring cells without antibody-IR700 conjugates were not injured at all. The antibody-IR700 conjugate can selectively destroy not only cells in vitro but also target cells in vivo in space and time.^[6]

Aggregation by photochemical reaction of IR700 and detection of released products by mass spectrometry

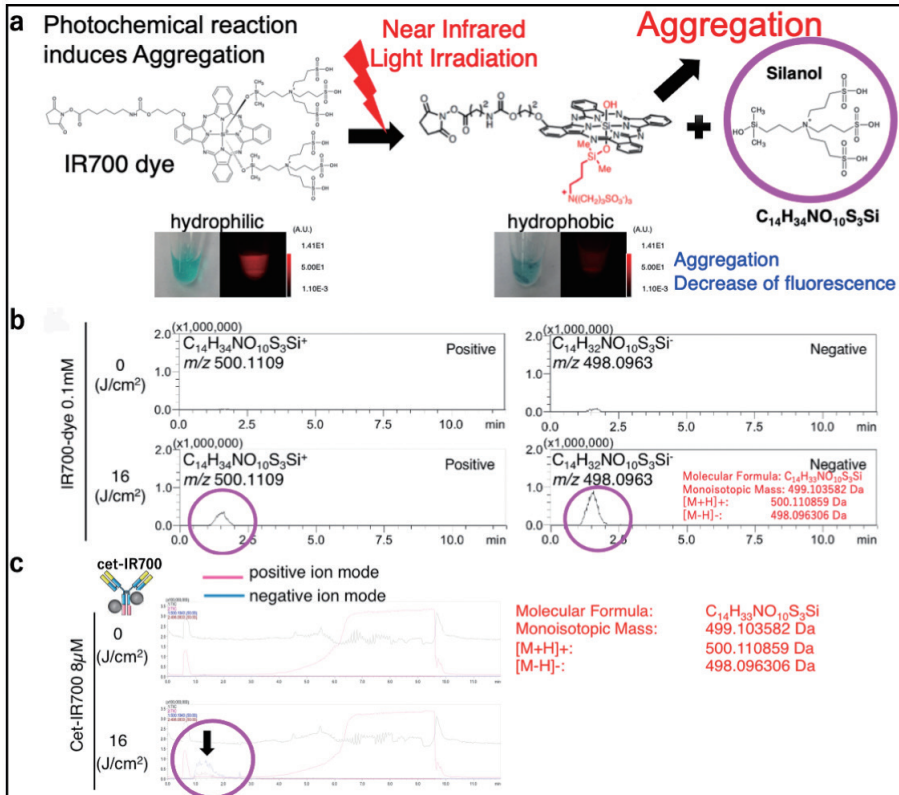


Figure 2 Aggregation by photochemical reaction of IR700 and detection of released products by mass spectrometry

- a. The photochemical reaction of IR700 as the starting point of NIR-PIT action: the silanol group attached to the silicon of the silicon phthalocyanine ring of IR700 is released by near-infrared light irradiation, and as a result, the hydrophilic nature of IR700 suddenly becomes hydrophobic and aggregates. As a result of aggregation, the fluorescence is lost.
- b. The silanol groups released from the supernatant after irradiation were detected by mass spectrometry (IT-TOF MS), proving the reaction.
- c. IT-TOF-MS analysis showed a peak of silanol group only at 16 J/cm² near-infrared light irradiation. By product scan (MS3) analysis, the structure was determined and proved to be silanol group.

Originally, IR700 dye (IR700 DX) was developed and marketed with the merit that its fluorescence hardly fades compared to other dyes (AlexaFluor680, etc.) during the number of scans in the fluorescence observation environment of microscope slide samples. However, the accumulation of *in vitro* and *in vivo* experiments with NIR-PIT clearly showed that the fluorescence of IR700 faded, which was a contradiction. As a hypothesis to resolve this discrepancy, we hypothesized a change in the chemical nature of IR700 due to photochemical reactions. The hypothesis is that the chemical nature (hydrophilic) of IR700 is rapidly changed to hydrophobic by the release of the silanol side chain by near-infrared light. The hydrophobized IR700 forms aggregates in the aqueous solution, and as a result of the aggregation, the fluorescence is lost from the solution in a series of photochemical reactions (Figure 2a)^[7]. It was thought that the aforementioned discrepancy could be explained by the difference between the inclusion state of the slide samples using non-water-soluble inclusion material and the water-rich biological environment.

Based on these hypotheses, in order to prove this mechanism, we attempted to measure the silanol groups (molecular weight 500) above and below the silicon phthalocyanine ring of IR700, which are thought to be released upon aggregation, by mass spectrometry. IT-TOF-MS was able to detect the peak of the molecular weight only in the case of light irradiation (Figure 2b). In addition, it has been found that this liberation reaction is enhanced in the

absence of oxygen and in an environment rich in electron donors, while the presence of electron absorbers inhibits the reaction. This indicates that this reaction is independent of oxygen and free radicals, and has the advantage that the reaction proceeds more easily in tumors under hypoxic conditions.

Next, in order to prove that the above reaction also occurred in the antibody-IR700 adduct, cetuximab-IR700 (cet-IR700) was irradiated in the near-infrared to prove that silanol could be measured and detected by mass spectrometry. In addition, the solution of cet-IR700 was confirmed by the product scan method to be of IR700 origin, not antibody origin, with the structure of the silanol group (Figure 2c).

SDS-PAGE analysis was performed to study the effect of photochemical reaction of IR700 on antibody IR700 adduct and antibody IR700 adduct-antigen protein complex. First, we found that NIR light irradiation (peak 690 nm NIR light laser irradiation) caused the antibody (cetuximab) band to smear on SDS-PAGE, resulting in the loss of IR700 fluorescence. The photochemical reaction of IR700 resulted in the protein denaturation, i.e., aggregation of the added antibody proteins (Figure 3a). As shown in the figure, the denaturation increased in a near-infrared light-dependent manner, and the aggregation was almost complete at 64 J/cm². It is also clear that the near-infrared fluorescence is lost due to aggregation. In order to investigate the effect

Examination of protein aggregation and loss of fluorescence of cet-IR700 by near-infrared irradiation and changes in the antibody-IR700 adduct-antigen protein complex using SDS-PAGE

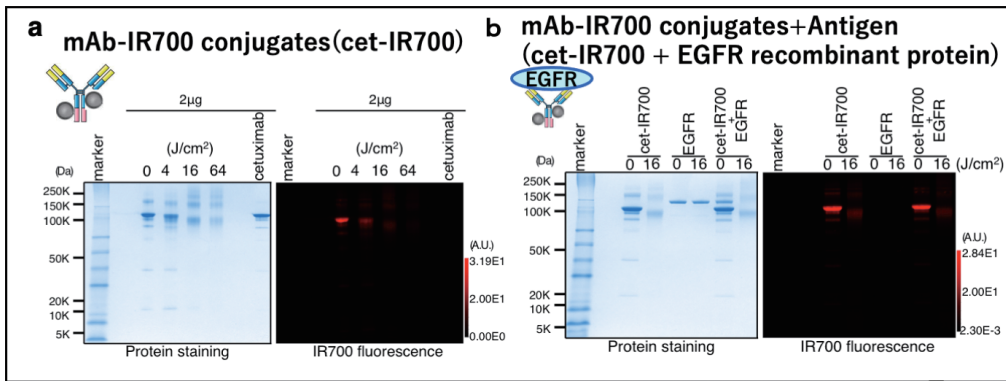


Figure 3 Examination of protein aggregation and loss of fluorescence of cet-IR700 by near-infrared irradiation and changes in the antibody-IR700 adduct-antigen protein complex using SDS-PAGE
 a. The band of cet-IR700 became a smear in a light-dependent manner and lost its fluorescence, indicating that it was aggregated.
 b. When cet-IR700 was bound to an antigen protein (EGFR) and irradiated with near-infrared light, the band of the antigen protein also became smeared, indicating that not only cet-IR700 but also its binding partner antigen was aggregated. With aggregation, fluorescence was also lost.

of the photochemical change of cet-IR700 on the binding partner antigen protein, we conducted a near-infrared irradiation experiment with cet-IR700 bound to EGFR protein (antibody-IR700 adduct-antigen protein complex) (peak 690 nm near-infrared laser irradiation measured at 16 J/cm²). SDS-PAGE showed that not only cetuximab but also EGFR protein, the antigen, was aggregated (Figure 3b). In addition, the loss of NIR fluorescence of IR700 was observed. From the above, it was confirmed that the complex of antibody IR700 adduct and antigen was aggregated by light irradiation. This indicates that the photochemical aggregation of IR700 causes the antibody to aggregate and also causes the antigen protein, the binding

partner of the antibody, to aggregate.

Although we were able to prove the photochemical reaction by mass spectrometry above, it is necessary to capture the change visually. This mechanism can be clarified by imaging the changes in a single antibody molecule and quantitatively measuring the photochemical reaction. Therefore, we attempted to perform nano-imaging in liquid by atomic force microscopy (AFM). Panitumumab-IR700 (pan-IR700) was adhered to a mica substrate and the top of the substrate was filled with 10 mM phosphate buffer containing 50 mM MgCl₂ for nano-imaging in liquid. Macroscopic views of 1.0 μm and microscopic images of

Observation and Quantification of Changes in panitumumab-IR700 (pan-IR700) under Near-Infrared Light Irradiation by Nano-Imaging in Liquid Using FM-AFM

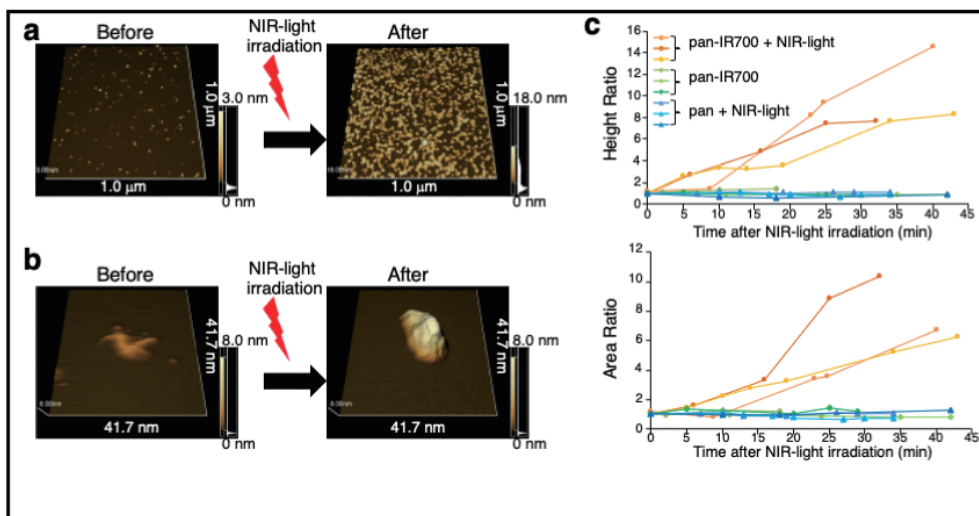


Figure 4 Observation and Quantification of Changes in panitumumab-IR700 (pan-IR700) under Near-Infrared Light Irradiation by Nano-Imaging in Liquid Using FM-AFM The changes of pan-IR700 before and after near-infrared light irradiation were investigated by nano - imaging in liquid using FM-AFM.
 a. The pan-IR700 molecules depicted on the mica substrate aggregated and became larger after light irradiation, and adhered to the mica substrate.
 b. pan-IR700 molecules depicted on the mica substrate have a Y-shape, but they aggregate and completely change their structure after light irradiation.
 c. Results of change quantification by liquid nano-imaging using FM-AFM; the height and area significantly increased only in the group where Pan-IR700 was irradiated with NIR-light.

Relationship of IR700 fluorescence and in vitro cell death, in vivo anti-tumor effects, respectively

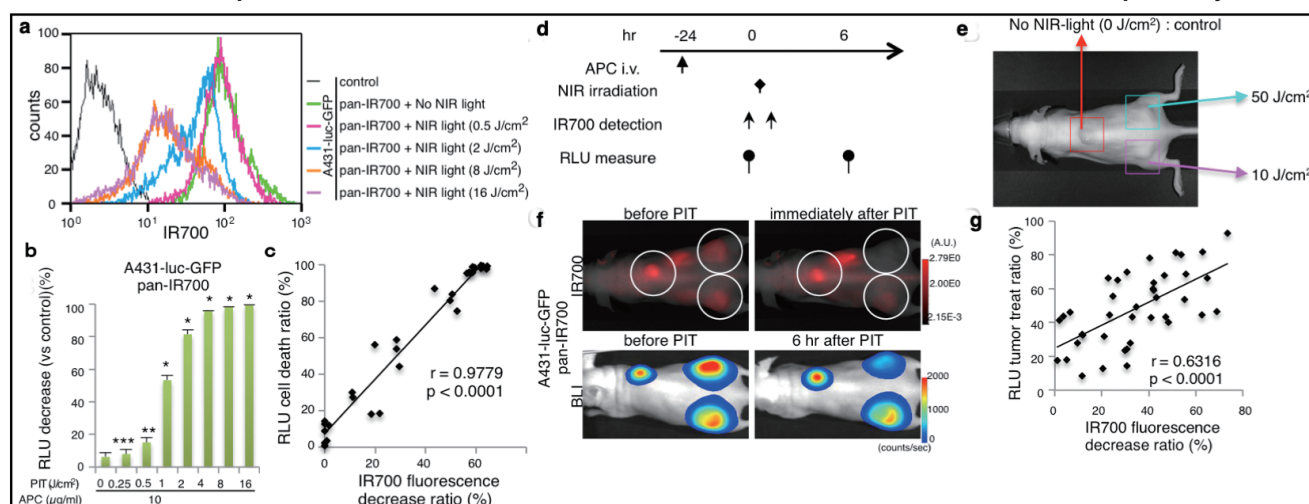


Figure 5 Relationship of IR700 fluorescence and in vitro cell death, in vivo anti-tumor effects, respectively
 a. IR700-fluorescence in vitro was detected before and after NIR-PIT with flow cytometry.
 b. Cytotoxicity in vitro was detected before and after NIR-PIT with luciferase activities.
 c. Positive correlations between decrease of IR700 fluorescence and cytotoxicity is detected ($r = 0.9779$).
 d. in vivo NIR-PIT regimen is showed.
 e. Mouse tumor inoculation outline and light dose.
 f. Representative image of treated mice.
 g. Positive correlations between decrease of IR700 fluorescence and anti-tumor effects is detected ($r = 0.6316$).

41.7 nm were taken. In the macroscopic image, a single molecule of pan-IR700 attached to the mica substrate was agglomerated and expanded as an oligomer by near-infrared light irradiation (measured value: 16 J/cm²). In the micro-image, a single molecule of pan-IR700 in the shape of a Y-shape underwent structural changes upon light irradiation, and expanded, deformed, and aggregated as an oligomer (Figure 4a, b). As a control, pan-IR700 alone (no light irradiation) and antibody (pan) plus light irradiation (16 J/cm²) were tested in the same way for quantification. As a result of quantification of the height and Area values over time, only the combination of pan-IR700 and light irradiation (16 J/cm²) was found to be dominant, with the height and Area values expanding over time (Figure 4c). Therefore, nano-imaging confirmed that the antibody-IR700 agglomerates upon near-infrared light irradiation.

In order to analyze the relationship between photochemical reactions and cell death in vitro, we analyzed the relationship between the loss of IR700 fluorescence on the cell membrane before and after light irradiation and cell death. It is known that near-infrared fluorescence is lost in a dose-dependent manner, and measuring the near-infrared fluorescence of IR700 in IR700 antibody adducts was thought to reflect the degree of protein aggregation. Changes in near-infrared fluorescence were quantified by flow cytometry (Figure 5a), and cell death was quantified by luciferase activity (Figure 5b), and the respective changes were analyzed as a scatter plot. The results showed a positive correlation between the decrease in IR700 fluorescence and the increase in cell death ($r = 0.9779$) (Figure 5c). This result suggests that cell death can

be measured from the amount of decrease in near-infrared fluorescence in target cells bound with IR700 antibody adducts, proving that IR700 photochemistry is strongly associated with cell death in NIR-PIT.

To analyze the relationship between the photochemical reaction and the in vivo anti-tumor effect, we analyzed the relationship between the loss of IR700 fluorescence in tumors before and after light irradiation and the anti-tumor effect (Figure 5d). As shown in Figure 5e, three tumors were created in the same individual and irradiated with 0, 50, and 100 J/cm² of near-infrared light, respectively, and the amount of change was measured. The change in IR700 fluorescence was quantified by the 700 nm channel of the PEARL imager, and the anti-tumor effect was quantified by the luciferase activity, which was analyzed in relation to the scatter plot (Figure 5f). The results showed a positive correlation between the decrease in IR700 fluorescence and the increase in antitumor effect ($r = 0.6316$) (Figure 5g). This result suggests that the anti-tumor effect can be measured from the amount of decrease in IR700 fluorescence (700 nm) across the target tumor bound with IR700 antibody adducts, proving that the photochemical reaction of IR700 is strongly related to the anti-tumor effect of NIR-PIT. Since this decrease in IR700 fluorescence may predict therapeutic efficacy, optimization of real-time imaging of IR700 fluorescence is underway.

Conclusion

The above results suggest that the initiation point of cell death in NIR-PIT is the hydrophobization of IR700 by

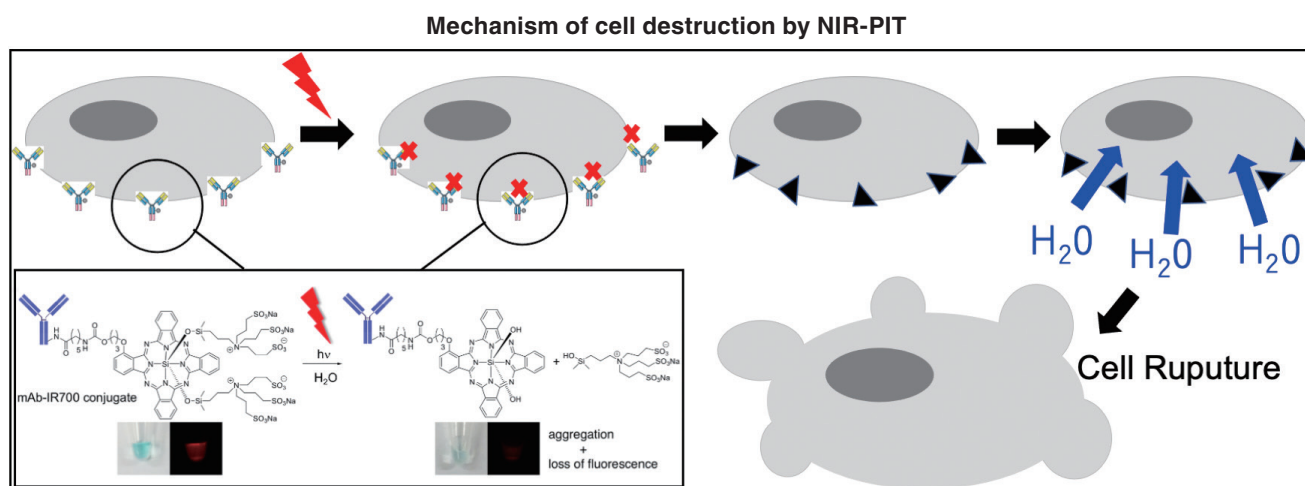


Figure 6 Mechanism of cell destruction by NIR-PIT

The antibody-IR700 complex binds to cancer-specific antigens on the cell surface. When the cells are irradiated with near-infrared light, the silanol group is released from IR700 and rapid hydrophobization occurs, resulting in the formation of aggregates including antibody and antigen proteins. This causes physical damage to the cell membrane. The osmotic disparity causes water to flow into the cell from outside the cell, causing the cell to rupture. Thus, the cell contents are disseminated to the surrounding area as necrosis to produce active cell destruction.

near-infrared light irradiation and the subsequent aggregation of antibody and antigen proteins^[7]. In this study, the aggregation of antibody-IR700 adducts by near-infrared light irradiation was revealed by nano-imaging, and the results were convincing enough to propose a new concept of cell death in NIR-PIT. Integrating these analytical chemical data, cell culture, and animal studies, the authors propose the following mechanism of action of NIR-PIT (Figure 6). The antibody-IR700 complex, which binds to cancer-specific antigens on cell membranes, suddenly aggregates upon NIR irradiation and acts as a physical cell membrane barrier by denaturing the cell membrane antigen to which it binds. This mechanism was considered to be a novel photocell death that is completely different from previous concepts. The clarification of this mechanism proved the superiority of NIR-PIT as a new anti-cancer modality, and led to its limited approval in Japan as a “fifth cancer treatment” ahead of other countries. In addition, as a further application, it is thought that it is possible to predict the effect of treatment in real time by measuring and evaluating near-infrared fluorescence before and after treatment as described in this paper, and devices are being developed that apply intraoperative fluorescence imaging devices. We look forward to the development of future applications.

* Editorial note: This content is based on HORIBA’s investigation at the year of issue unless otherwise stated

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