Development of a New In Vivo Optical Probe for Biological Diagnosis and Therapy

Michitaka Ozaki, Takeaki Ozawa, and Yuma Yamada

Abstract Real-time imaging of the specific markers of lesions in the living body will provide valuable information in various physiopathological situations. Clinically, real-time imaging will definitely aid accurate diagnosis and rational therapy, especially in the surgical field.

In this chapter, we describe some unique optical probes for "biological imaging" and our recent challenge in undertaking development of a new type of in vivo probe. The reduction-oxidation-sensitive green fluorescent protein (roGFP) and bioluminescent luciferase probe for caspase-3 activity have been useful for understanding of the dynamic changes of liver redox states and apoptotic cell death. To overcome the difficulty of imaging in deeper lesions by optical probes, we newly developed a far-red bioluminescent probe. Lastly, we have undertaken the challenge to develop an innovative optical probe that switches "on" only when the probe recognizes a target molecule to reduce non-specific signals in vivo. The project of developing this unique probe is still underway.

Regarding a carrying system of the probe into cells in vivo, we have developed a liposome with cell-penetrating octa-arginine peptides (R8) and a pH-sensitive fusogenic peptide (GALA), which delivers the functional proteins into cells efficiently and rapidly in vivo.

We believe that these optical probes will provide a new avenue toward new diagnosis and therapy to come in the future.

Keywords Optical probe • In vivo imaging • Fluorescence • Bioluminescence • Oxidative stress • Apoptosis • Liver • Liposome

M. Ozaki, M.D., Ph.D. (✉)
Laboratory of Molecular and Functional Bio-Imaging (LMFBI), Faculty of Health Sciences, Graduate School of Health Sciences, Hokkaido University,
N-12, W-5, Kita-ku, Sapporo, Hokkaido 060-0812, Japan
e-mail: ozaki-m@med.hokudai.ac.jp

T. Ozawa
Department of Chemistry, School of Science, The University of Tokyo,
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Y. Yamada
Laboratory for Molecular Design of Pharmaceutics, Faculty of Pharmaceutical Sciences, Hokkaido University, N-12, W-5, Kita-ku, Sapporo, Hokkaido 060-0812, Japan

Abbreviations

Ad*pcFluc*	Adenovirus vector encoding *pcFluc-DEVD*
Dlk1	Human Delta-like protein
AP-1	Activator protein 1
ATP	Adenosine triphosphate
BG	Benzylguanine
BRET	Bioluminescence resonance energy transfer
CLuc	Cypridina luciferase
DEVD	Asp-Glu-Val-Asp (a substrate sequence of caspase-3)
FBP	Far-red bioluminescent protein
FLuc	Firefly luciferase
FlucC-SNAP	C-terminal fragment of luciferase conjugated with SNAP
FlucN-SNAP	N-terminal fragment of luciferase conjugated with SNAP
H/R	Hypoxia and reoxygenation
I/R	Ischemia and reperfusion
MAPK	Mitogen-activated Protein Kinase
MEND	Multi-functional envelope-type nano device
NF-kB	Nuclear factor-kappa B
OS	Oxidative stress
pc*Fluc-DEVD*	Cyclic luciferase reflecting caspase-3 activity
PET	Positron emission tomography
roGFP	Reduction-oxidation sensitive green fluorescent protein
ROS	Reactive oxygen species
R8	Octa-arginine peptide
SNAP	A 182 residues polypeptide that can be fused to any protein of interest

Introduction

Real-time monitoring of molecular markers of lesions in the living body will provide valuable information on various physiopathological phenomena. This will aid understanding of lesions qualitatively as well as quantitatively. Clinically, this will lead to more accurate diagnosis and rational therapy. Especially in the surgical field, this kind of technology will enable us to "see" the biological characteristics (and potential pathological/progressive behavior) of the lesions during operation as well as its location, size, and expansion, which will definitely help the surgeon select the best surgical procedure for the patient.

The conventional imaging technologies commonly used in clinical practice include CT, PET-CT, and MRI. These technologies provide useful and geographical information about the location, size, shape, and expansion (including distant metastasis), and aid accurate clinical diagnosis and pre-operative staging. However, they

Table 1 Comparison between conventional imaging technology and optical imaging

Type of imaging	Advantages	Disadvantages
Conventional imaging technology (CT/ PET-CT/MRI)	(1) Can image exact size of lesions macroscopically	(1) Does not provide information on biological features of lesions
	(2) Can image whole body	(2) Does not show exact locations during operation
	(3) Enables preoperative diagnosis and clinical staging	(3) High cost and space occupying
Optical imaging (fluorescence/ bioluminescence)	(1) Can image molecules/cells of interest; enables biological imaging of lesions	(1) Not good at examining deep lesions
	(2) Low cost and space saving; enables intra-operative real-time imaging	(2) Needs fluorescent/ bioluminescent reagents for imaging
		(3) Relative estimation

do not show the biological characteristics of the lesion, nor its exact location during the operation. In addition, they require a lot of space and much expense for installation.

On the other hand, optical imaging by fluorescence or bioluminescence will provide biological information with sophisticated probes at low cost and in a small space. Disadvantages of optical imaging are that it requires a customized probe (fluorescence or bioluminescence) and has difficulty in imaging deeper lesions. In addition, signals from optical probes are relative, not absolute (Table 1).

An Optical Probe for In Vivo Imaging of Caspase-3 Activity

Many optical probes have been developed to visualize protease activities for biological in vivo imaging [1–7]. They have been used to analyze the dynamic changes in the cell and organ stresses and injuries, including hypoxia and reoxygenation (H/R) and ischemia and reperfusion (I/R) of cells and organs, respectively. I/R-induced injury is an important concern in various clinical situations, including surgical resection of organs, transplantation of organs and cells, and myocardial/ cerebral/intestinal infarction. In these clinical situations, prolonged ischemia followed by reperfusion results in extended organ injury caused by apoptosis, necrosis, and organ failure [8]. Although the mechanisms of I/R-induced injury are complex, post-ischemic apoptotic and/or necrotic injury play a pivotal role in subsequent organ failure [9, 10]. Therefore, noninvasive monitoring of cell and organ injury (e.g., caspase activity) in vivo is definitely beneficial and is able to provide important clues for treatment.

We previously developed a novel probe of cyclic luciferase reflecting caspase-3 activity (pc*Fluc-DEVD*) [2]. Two fragments of DnaE inteins are fused with neighboring N-terminal and C-terminal ends of firefly luciferase, which are connected
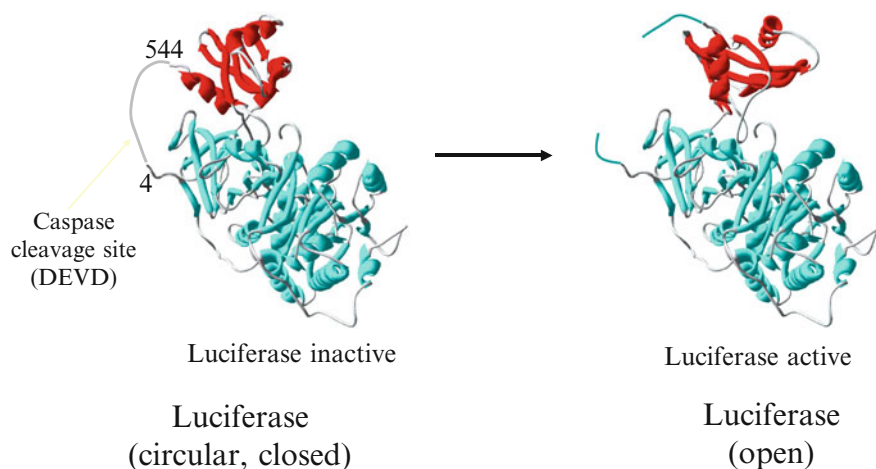
with DEVD (Asp–Glu–Val–Asp [a substrate sequence of caspase-3]). After translation into a single polypeptide, the N-terminal and C-terminal ends of the luciferase are ligated by protein splicing, producing a closed circular polypeptide chain. The cyclic luciferase structure is distorted and therefore almost loses its bioluminescent activity. When caspase-3 is activated in cells, the DEVD chain is cleaved, which restores firefly luciferase (Fluc) luminescent activity by returning to the original structure (Fig. 1) [11].

This cyclic luciferase was first applied for quantitative measurement of caspase-3 activity in living cells. HeLa cells transfected with this probe were stimulated with staurosporine, an inducer of apoptosis. This bioluminescent probe based on cyclic luciferase allowed precise and quantitative measurements of caspase-3 activity in living cells. Moreover, the response of this cyclic luciferase to caspase-3 activation was rapid and paralleled biochemically assessed apoptosis.

This imaging technology by cyclic luciferase could be applied for in vivo real-time imaging of caspase-3 activity in living mice. We successfully imaged the caspase-3 activity in post-ischemic liver using our caspase-3 probe (Fig. 2). By transfecting the adenovirus vector encoding *pcFluc-DEVD* (Ad*pcFluc*), we investigated whether or not this probe could monitor caspase-3 dependent apoptosis in the mouse liver during I/R (Fig. 2) [11]. We confirmed the efficacy of the probe in detecting apoptotic liver injury in a mouse model, which showed the potency for future clinical application.
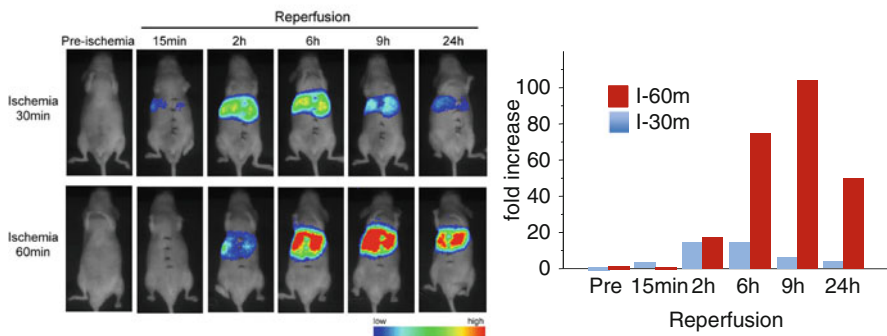

Fig. 1 Bioluminescent probe for caspase-3 activity. The principle for monitoring activity of caspase-3 is based on cyclic firefly luciferase (Modified from Ozaki et al. [11], with permission)

## *In vivo* imaging of caspase-3 activity by the optic probe

Fig. 2 In vivo imaging of caspase-3 activity by the optical probe. An ischemic insult to the liver induced caspase-3 activation (Modified from Ozaki et al.[11], with permission)

An Optical Probe for In Vivo Imaging of the Hepatic Redox State

Reactive oxygen species (ROS) generated during I/R of organs certainly play a pivotal role in I/R-induced organ injury and failure [12]. Hepatic I/R results in massive cell death (a) through apoptosis and necrosis mediated by excessive generation of ROS in hepatocytes early after I/R [13]; and (b) through necrosis caused by neutrophilic infiltration in the later phase [14]. According to the numerous studies on liver I/R in mice, organ injury in the early post-I/R period is definitely mediated by generation of ROS in hepatocytes [15, 16]. Excessive ROS directly exert deleterious effects on cells and tissues through lipid peroxidation, protein degradation, and DNA damage. In addition, redox-sensitive molecules, such as nuclear factor-kappa B (NF-κB), activator protein 1 (AP-1), and some mitogen-activated protein kinases (MAPKs), are potentially activated under these oxidative conditions and play pivotal roles in I/R-induced injury [17]. Therefore, monitoring cellular redox states by visualizing cellular ROS in post-I/R tissue has been one of the possible strategies for forecasting I/R-induced injury and failure [18]. These considerations suggest that the post-I/R redox state may be a good prognostic indicator of liver injury and function after liver surgery and transplantation, implying that in vivo monitoring may provide more diagnostic and therapeutic options in clinical liver surgery and transplantation.

For in vivo assays of tissue oxidative stress (OS), tissues are usually homogenized and subsequently assayed, either with redox-sensing electrodes or by measuring the ratios of the reduced and oxidized forms of glutathione and ascorbate. Recently, the redox states of tissues have also become assessable by using the dyes 5- (and 6-) carboxy-2,7-dichlorodihydrofluorescein diacetate [19] and dihydrofluorescein diace-

In vivo imaging of I/R-induced oxidative stress of mouse liver
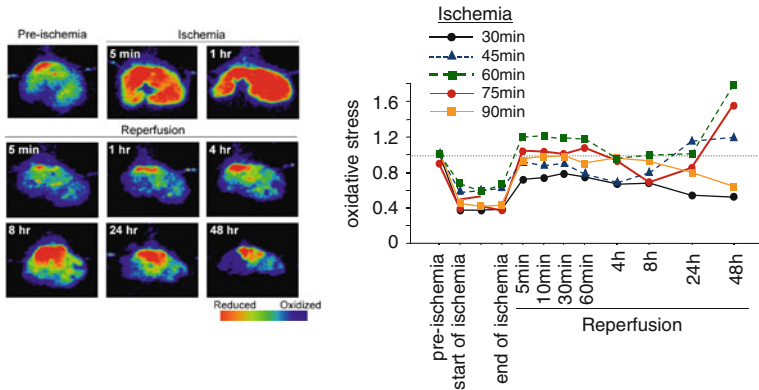
Fig. 3 In vivo imaging of I/R-induced oxidative stress of the mouse liver. The roGFP probe showed dynamic changes in liver redox states. The *left panel* depicts representative images of the dynamic changes after 60 min of ischemia, followed by reperfusion (oxidation, *green to blue*; reduction, *orange to red*) (Modified from Haga et al. [10], with permission)

tate [20]. Although such approaches allow evaluation of the sum of the oxidized and reduced species present, and thereby estimation of the overall redox state of the tissue, these techniques do not allow measurement of redox potentials in real time. More importantly, homogenization of tissues precludes the possibility of monitoring dynamic changes in OS in the same animal and organ.

A newly developed reduction-oxidation-sensitive green fluorescent protein (roGFP) probe (GFP with mutations of C48S, S147C, Q204C, and S65T) [1] could be applied for in vivo imaging. Use of the roGFP probe enables evaluation of cell and organ redox states non-invasively and continuously. Adenovirally transduced roGFP in the mouse liver was shown to act as a good indicator of pre- and post-ischemic liver redox states (Fig. 3) [10]. Through use of this roGFP probe, post-operative liver injury may be predicted by real-time monitoring of liver redox states.

Optical Imaging of Deeper Lesions in the Living Body

An increasing number of antibodies have been applied for targeting antigens on the surface of cancer cells for clinical diagnosis and therapy, on the basis of the fact that some specific antigens expressed on the cancer cell surface reflect malignant behaviors including invasion, metastasis, and neo-vascularization [21–23]. Real-time visualization of tumor-specific antigens in the living body will enable timely understanding of cancer expansion and its biological behaviors, and prediction of the patient's prognosis in the near future.

Fluorescence and bioluminescence imaging technologies have played important roles in molecular imaging in cells and small animals [24–26]. Photon detection is easy to use, cost saving, and space saving, compared with radioisotope imaging. Basically, bioluminescence imaging is achieved with a luciferin–luciferase reaction in the presence of sufficient amounts of molecular oxygen and adenosine triphosphate (ATP). However, most bioluminescence spectra are observed in the visible region, which overlaps with the absorption spectra of hemoglobin and tissue constituents, therefore attenuating the bioluminescence intensity in living animals.

Recently, a self-illuminating quantum dot probe was developed to improve the light penetration based on bioluminescence resonance energy transfer (BRET) between the bioluminescence of *Renilla* luciferase and quantum dots [27]. The multivalent conjugation of *Renilla* luciferase to single dots allowed for highly efficient BRET between luciferase and quantum dots. However, the large size of the conjugate caused problems in metabolism and localization in vivo [28]. Several far-red fluorescent protein variants showing maximal emission at around 650 nm have been developed for in vivo imaging [29], but are not well characterized as energy acceptors for BRET systems. On the other hand, the organic dye indocyanine and its derivatives have molecular weights of less than 1,200 Da; they produce far-red fluorescence and have been widely used for in vivo imaging [30].

Luciferase conjugated to such organic dyes is expected to create new possibilities for in vivo application. *Cypridina* luciferase (CLuc) catalyzes the oxidation of *Cypridina* luciferin to yield light emission peaking at 460 nm [31].

The luciferase genes from both *Cypridina hilgendorfii* and *C. noctiluca* have been cloned [32], the latter of which we used. This self-secretory CLuc of 62 kDa has some unique properties as a bioluminescent enzyme [33]. The secreted protein contains 17 disulfide bond pairs and is highly stable under physiological conditions. Its turnover rate (1400 luciferin molecules per minute) is the highest among known luciferases [34]. We have expressed recombinant CLuc in yeast and applied it to ELISA. We conjugated a far-red fluorescent indocyanine derivative to biotinylated CLuc via glycol chains and named this far-red bioluminescent protein (FBP).

A monoclonal antibody against human Delta-like protein (anti-Dlk1), one of the embryonic antigens expressed on the surface of many cancer cells, including hepatocellular carcinoma, was then produced. Using anti-Dlk1 monoclonal antibody linked to FBP via biotin–avidin interaction, we achieved bioluminescence imaging of cancer cells in vivo in mice (Fig. 4).

However, very unfortunately, there were some non-specific signals from the tissues that did not express Dlk-1, though the tumor implanted into the liver itself was imaged clearly by this probe. This may have been because excessive and unbound antibodies circulating in the blood or captured in tissues emitted signals when the substrate was injected. In order to apply this probe to clinical application, non-specific signals must be reduced as much as possible. So, we have launched the following new project.

In vivo imaging by a new optic probe for deeper lesions in mouse
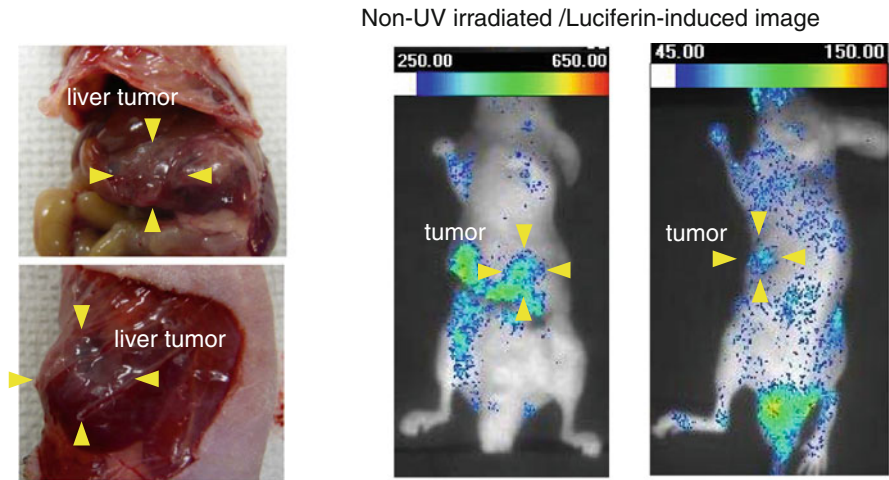
Non-UV irradiated /Luciferin-induced image

Image was obtained 24 hr after Luciferin adm (iv)

(400mg/200ml i.v.)

Fig. 4 In vivo imaging by a new optical probe for deeper lesions in the mouse. A liver tumor of the mouse was imaged, but some non-specific signals were observed, probably because of excessive and unbound antibodies

Development of an In Vivo Optical Probe for Antigen-Specific Imaging Inside/Outside Cells

For accurate biological diagnosis and rational therapy in clinical practice, we need to know the biological features of the lesion as well as its size, shape, and extent. For this purpose, we plan to develop a new optical probe, which will image the target molecules only after binding to the antigen. This does not allow unbound antibody to emit light signals, and therefore it will lead to the reduction of non-specific signals in vivo. Additionally, the probe should be applied to targets inside as well as outside cells in vivo, because there are lots of diagnostic and therapeutic targets inside as well as outside cells. In order to create a new type of in vivo optical probe, the following two technologies have to be developed (Fig. 5).

(A) Development of an Optical Probe Activated Only When it Binds to the Target Molecule

In order to develop a probe activated after binding to the target antigen in vivo, we have designed a new type of luciferase–antibody conjugated probe. We planned to prepare split firefly luciferases, the N-terminal and C-terminal fragments of luciferase conjugated with SNAP (a 182-residue polypeptide that can be fused to any
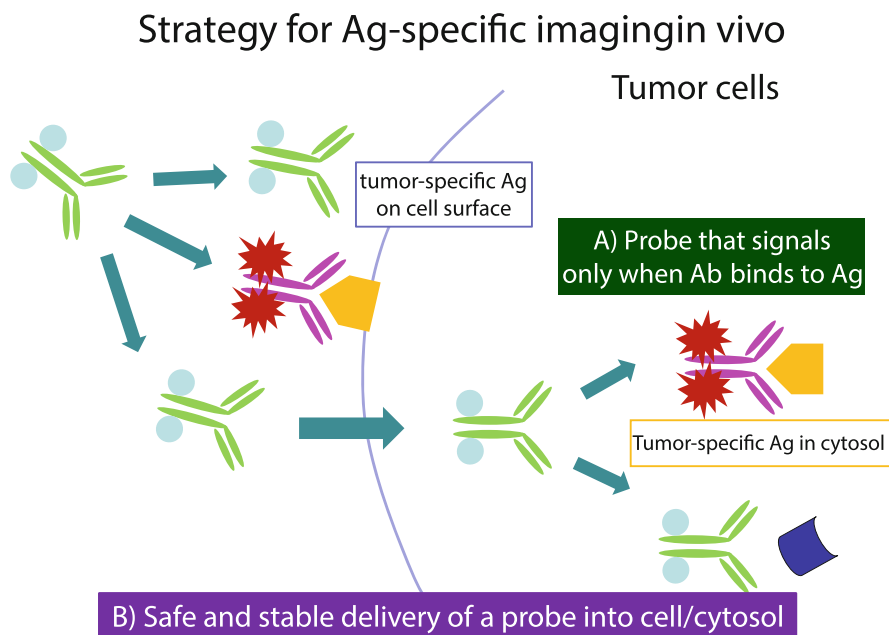
Fig. 5 Strategy for antigen-specific imaging in vivo

protein of interest)—FlucN-SNAP and FlucC-SNAP, respectively—and to prepare a polyclonal antibody covalently connected with benzylguanine (Ab-BG). When the antibodies with FlucN and FlucC recognize and bind to the close epitopes of the same antigen, split luciferase fragments will be reconstituted and activated (Fig. 6). Theoretically, the probe should enable us to see the target molecule in tissues and cells only when it exists.

We plan first to express FlucN-SNAP and FlucC-SNAP proteins by *Escherichia coli*, and then to bind benzylguanine (BG) with polyclonal Ab. Lastly, we will conjugate these proteins (Fig. 7).
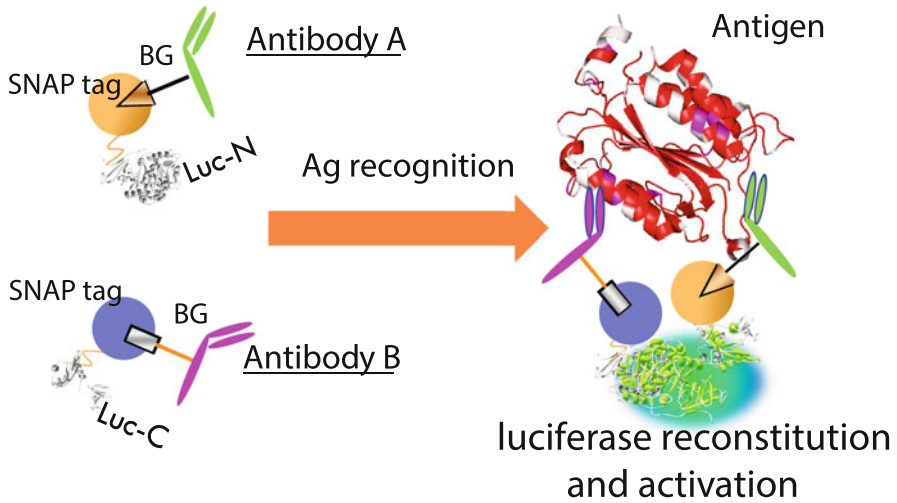
To date, we have succeeded in obtaining sufficient amounts of FlucN-SNAP and FlucC-SNAP proteins by the *E. coli* expression system, and we have confirmed the good binding capacity of FlucN-SNAP and FlucC-SNAP proteins to BG. So, we have bound BG with polyclonal Ab, and performed conjugation with Fluc-SNAPs. We are now examining the probe function in our in vitro system.

(B) Development of a Safe and Stable System for Delivery of Optical Probes into Cells

In order to develop a new system to deliver sufficient amounts of optical probe safely into cells in vivo, we are now developing a new liposome-based delivery system.

There seem to be two important processes for developing an effective and rapid delivery system (Fig. 8). First, the carrier including probes in it should be

Concept for antigen-dependent optic probe

BG
Antibody A
SNAP tag
Luc-N
Antigen
Ag recognition

SNAP tag
BG
Antibody B
Luc-C

luciferase reconstitution
and activation

Fig. 6 Concept for an antigen-dependent optical probe. When the probes with C-/N-terminal fragments of luciferase recognize the antigen, luciferase is reconstructed and activated
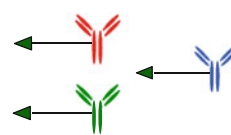
Procedures of making a probe

① Expression of FlucN-SNAP & FlucC-SNAP

| FlucN | SNAP | 6His |
| FlucC | SNAP | 6His |
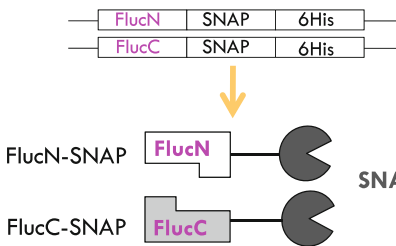
② Binding BG (Ligand) to polyclonal Ab

◀ : Benzylguanine group

FlucN-SNAP   FlucN

FlucC-SNAP   FlucC

SNAP-tagged

FlucN

FlucC

③ Conjugation of Fluc fragments and antibodies by SNAP-BG binding

Fig. 7 Procedure for making a probe: (1) expression of FlucN-SNAP and FlucC-SNAP; (2) binding BG (ligand) to polyclonal Ab; (3) conjugation of Fluc fragments and antibodies by SNAP-BG binding
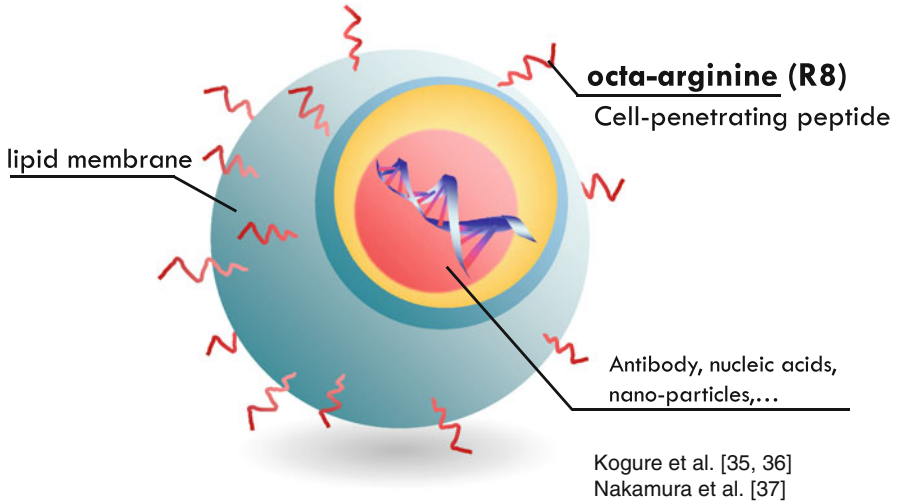
Strategy for cellular delivery of the probe
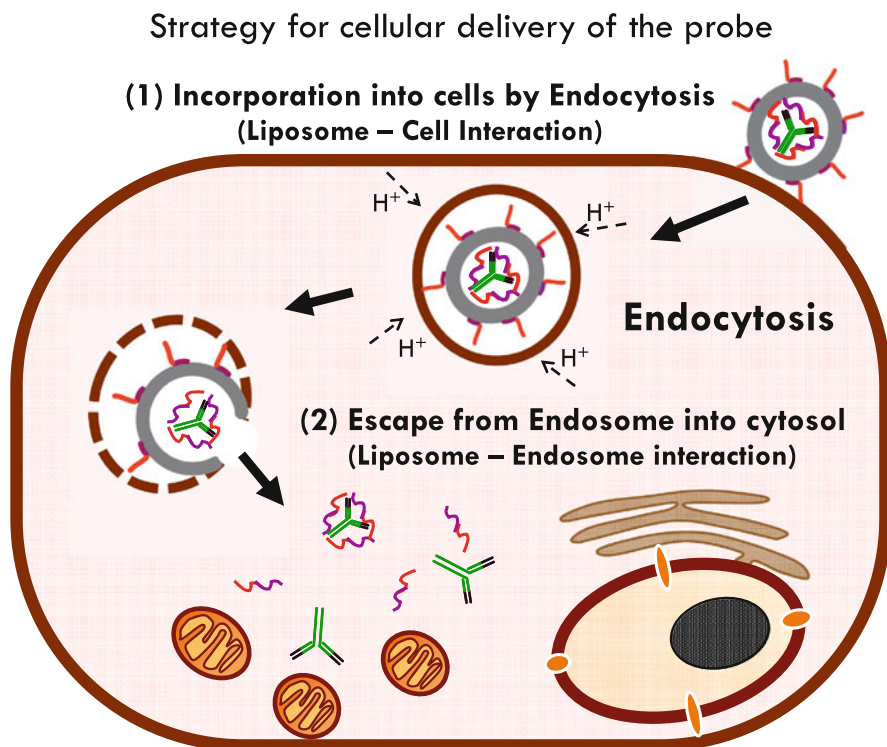
Fig. 8 Two important processes for efficient cellular delivery of probes: (1) incorporation into cells by endocytosis (a liposome–cell interaction); (2) escape from the endosome into the cytosol (a liposome–endosome interaction)

incorporated into cells sufficiently and rapidly. Second, the incorporated probe should be released from the lysosome into the cytosol. These processes should not be harmful to the cells and the probes.

We have already made a prototype liposome-based carrier for efficient drug delivery in vivo. We further added a cell-permeable octa-arginine peptide (R8) to the liposome, expecting better incorporation into cells (Fig. 9). In the in vitro experiments, the R8 liposome successfully delivered the "antibody" into the cells much better than the liposome without R8. The R8 liposome, however, failed to escape the "antibody" from the endosome into the cytosol.
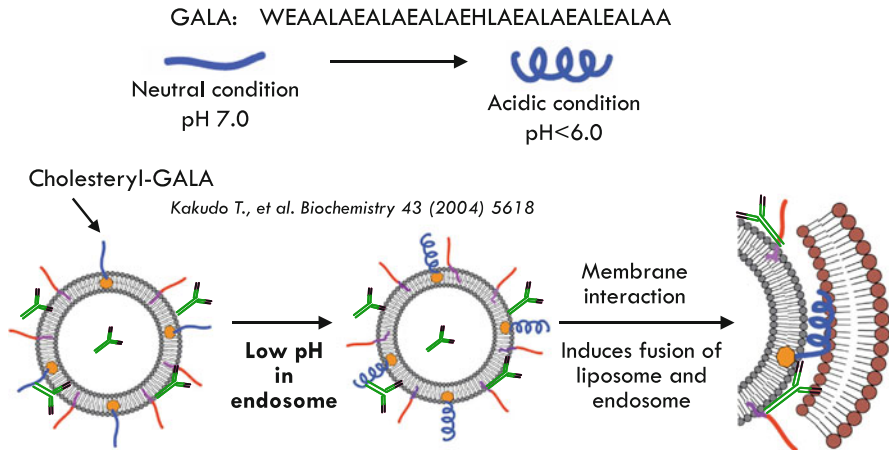
Next, we applied GALA to make the probe release rapidly and efficiently from the endosome into the cytosol (Fig. 10). GALA is a 30-amino-acid peptide with a glutamic acid–alanine–leucine–alanine repeat that contains a histidine and tryptophan residue. GALA changes its conformation from a random coil to an amphipathic alpha-helix when pH values are between 7.0 and 5.0. At neutral pH, GALA is water soluble, while at acidic pH, GALA binds to bilayer membranes. So it is expected to promote rapid fusion of the liposome with the endosomal membrane in the cell. The fusogenic liposome was modified with a cell-penetrating R8 and also GALA.

Multi-functional Envelope-type Nano Device (MEND) : R8-Liposome

octa-arginine (R8)

Cell-penetrating peptide

lipid membrane

Antibody, nucleic acids,
nano-particles,…

Kogure et al. [35, 36]
Nakamura et al. [37]

Fig. 9 Concept of a multifunctional envelope-type nano device (*MEND*). Octa-arginine (R8) is bound to the liposome surface (R8 liposome)

GALA: pH sensitive fusogenic peptide

GALA: WEAALAEALAEALAEHLAEALAEALEALAA

Neutral condition
pH 7.0

Acidic condition
pH<6.0

Cholesteryl-GALA

Kakudo T., et al. Biochemistry 43 (2004) 5618

Low pH
in
endosome

Membrane
interaction

Induces fusion of
liposome and
endosome

Fig. 10 The GALA pH-sensitive fusogenic peptide changes its conformation depending on pH values and is expected to promote lipid-membrane fusion

The presence of R8 enhanced the cellular uptake of antibodies, while the mounting of GALA reduced the endosomal entrapment, resulting in release of the antibody into the cytosol within 30 min in in vitro experiments. Moreover, compared with the commercially available reagents for protein delivery, the R8/GALA-modified liposome was superior in terms of both cellular uptake and transport into the cytosol. The specific antibody delivered by the R8/GALA liposome was detected by its epitope, which indicated that the R8/GALA liposome basically did not deteriorate the protein function.

Conclusion

In this chapter, we have described possible application of an optical probe for "biological imaging" and our recent challenge in undertaking development of a new type of in vivo probe.

In the mouse liver I/R model, the fluorescent probe, roGFP, and bioluminescent luciferase probe, pc*Fluc-DEVD,* were useful in understanding the dynamic changes of liver redox states and apoptotic cell death. We could understand the pathological significance of OS and apoptosis in I/R-induced liver injury. Because of the difficulty of imaging of deeper lesions by optical probes, we developed a unique probe loaded on an antibody. This probe successfully imaged a target molecule in deeper regions in mice, but also revealed another problem—an in vivo non-specific signal from excessive and unbound antibodies. Lastly, we undertook the challenge to develop an innovative optical probe which switches "on" only when the probe recognizes a target molecule. The project of developing this unique probe is still underway but seems very promising at this time.

Regarding a carrying system of the probe to target molecules in vivo, we developed a liposome bound with a cell-penetrating R8 and GALA. The R8/GALA liposome delivered the "antibody" to the cells and also the cytosol efficiently and rapidly in vivo.

In the future, we plan to prove the efficacy of the new probe carried by the R8/GALA liposome in in vivo experiments.

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