２０２３年度

青山学院大学審査学位論文

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Studies on artificial DNA for metabolite analysis and drug design

代謝物解析と薬剤設計を指向した人工核酸の開発に関する研究

理工学研究科

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Contents

General Introduction

Metabolite Analysis

Metabolites are the intermediate and final products of metabolic processes in living cell. Metabolites are close to phenotypes and change depending on the state of the cell. Therefore, metabolite analysis is very important for disease diagnosis and elucidation of pathophysiology. In recent years, several studies have conducted to investigate the correlation between genes and metabolites and diseases.[1] In fact, disease-related metabolites have been identified, and such metabolites are expected to serve as biomarkers of disease. For analyzing metabolites, liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis-mass spectrometry (CE-MS) are mainly used.[1,2] These methodologies are based on mass spectrometry and are preceded by separation operations of LC or CE before mass analysis. LC-MS has been used to analyze metabolites with high accuracy, and CE-MS has been realized to analyze metabolites in single cells. Tanaka and coworkers reported detection of 20 amino acids in single cell using micro needle and CE-MS (Figure 1).[2]

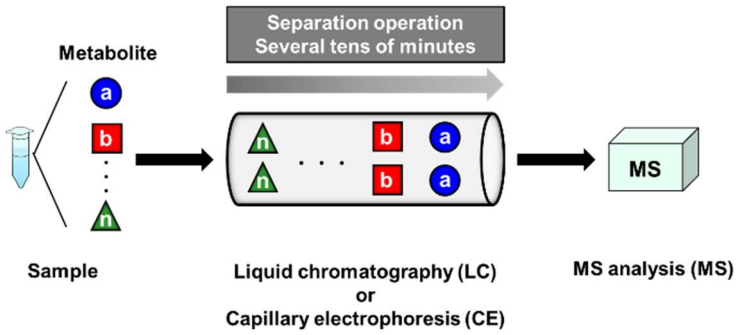

Figure 1. Metabolite analysis using LC-MS and CE-MS.

On the other hand, fluorescent probes for target metabolites are also developed. Using fluorescent probe, it is realized to visualize target metabolite in living cells.[3,4] Urano and coworkers developed fluorescent probes for glutathione (GSH).[4] This probe was FRET based fluorescent probe consisted of TAMRA and silicon rhodamine which reacted with nucleophiles such as GSH (Figure 2). After reaction with GSH, FRET efficiency changed and fluorescent signal from silicon rhodamine disappeared. These fluorescent probes were very useful for real-time monitoring of target metabolite.

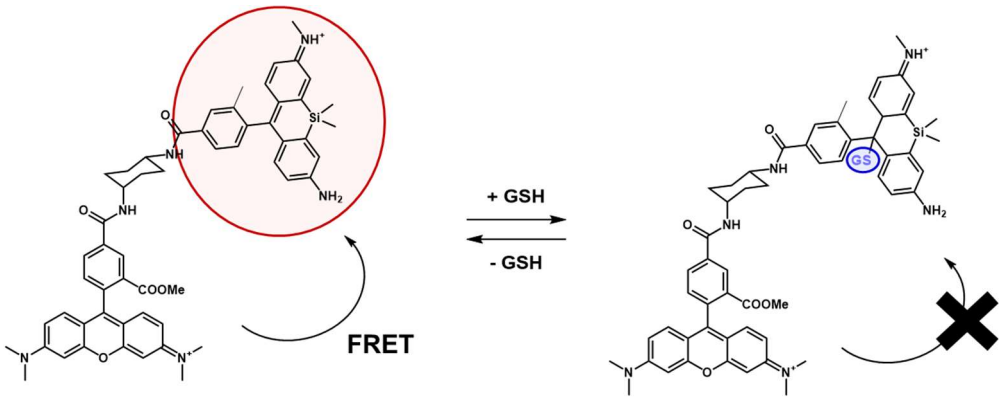

Figure 2. Fluorescent probe for GSH reported by Urano *et.al*.[4]

While these conventional methodologies can be used effectively, they have several drawbacks. The measurement of MS requires several tens of minutes of separation operations per sample, which requires a huge amount of time for the analysis of a large number of samples. Thus, low throughput is a main limitation for MS measurements. On the other hand, for the fluorescent method to identify metabolite, it is also difficult and time-consuming to design and synthesize the fluorescent dyes because they must be modified with functional groups corresponding to each target metabolite. In addition, the overlap in wavelengths of the fluorescent dyes limits the number of metabolites that could be detected simultaneously.

Metabolite analysis by means of functional DNAs

In order to construct a molecular system that easily identifies multiple metabolites, several DNA systems were employed in this thesis. Since DNA can be amplified by PCR and simultaneous high-throughput analysis using Next-Generation Sequencer (NGS) is possible,[5] we expected that transferring metabolite information to DNA sequences would be useful for simultaneous analysis of very small amounts of metabolites. As the DNA systems, functional oligodeoxynucletides (ODNs), DNA aptamers and DNA encoding system were utilized. In addition, we also employed biotin-avidin interaction to prepare complicated and sophisticated molecular system consisted of DNA, protein and small functional substituents. The individual functional molecules are outlined below.

Functional artificial oligonucleotides

Oligonucleotides could be synthesized automatically and modified by any molecules quite easily. Thus, many artificial oligonucleotides which have functional molecules in any places have been reported.[6,7,8,9] Firstly, functional oligonucleotides could be used as drugs or their carrier molecules. Mirkin and coworkers developed spherical nucleic acid (SNA) conjugates consisted of gold nanoparticle and thiol modified DNA.[6] SNA have high cellular uptake and stability to act as nucleic acid medicine to show selective cytotoxic effects. On the other hand, our laboratory reported DNA aggregates using amphiphilic oligonucleotides, which consisted of hydrophobic alkyl chain (Figure 3).[7] These amphiphilic oligonucleotides aggregated in aqueous solution like micelle, called DNA aggregates, which could incorporate hydrophobic drugs inside and transport them into cells through endocytosis pathway.

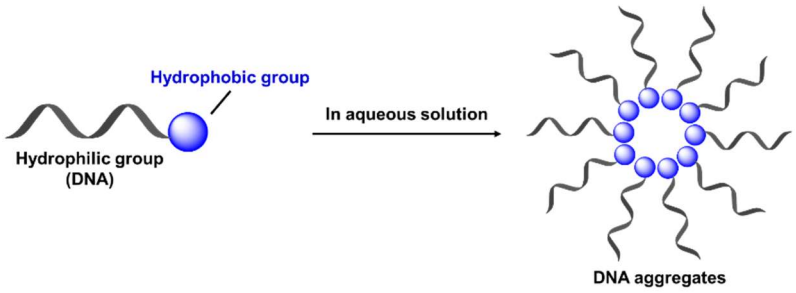

Figure 3. Formation of DNA aggregates using amphiphilic oligonucleotide.

Next, oligonucletides which is modified by fluorescent probe is used as molecular probe for target nucleic acids. For example, oligonucleotides that formed duplexes with the target gene have been used for their detection, and this method is called fluorescence in situ hybridization (FISH).[8] By using oligonucleotides with fluorophore modification, gene mapping and visualization of genetic abnormalities were successfully achieved. Modified oligonucleotides were also used for visualization of target protein. Kashida and coworkers reported multi-color target antigen imaging using biotinylated oligonucleotide and streptavidin modified polystyrene beads.[9] This methodology could realize multi antibodies imaging by using duplex formation of DNA. Thus, functional oligonucleotides work as useful biomaterial with high biocompatibility.

Nucleic acid aptamers

Nucleic acid aptamers are DNA or RNA oligomers that recognize target molecules. These oligomers could be sorted by systematic evolution of ligands by exponential enrichment (SELEX) method and are easily prepared (Figure 5).[10] Taking advantage of the selective binding of these aptamers to proteins, aptamers that bind to antigens on the cell membrane surface can be used to recognize and label target cells. In addition, these aptamers have been used for imaging and drug delivery of target cells.[11,12] Yang and coworkers achieved cancer cells imaging and capturing by using DNA aptamer for

epitherial adhesion molecule (EpCAM), which is expressed in cancer cell line.[12] Also, DNA aptamer and drug conjugate have been used for target cell therapy. Thus, aptamers have been used as a variety of biomaterials due to their usefulness.

DNA encoding system

Molecular information could be encoded into DNA sequence information. One of the most well-studied applications of DNA encoding system is shown in Figure 4.[13] A low-molecular-weight compound that serves as a substrate for a protein is modified with a DNA strand to create a substrate for a protein. The resulting DNA strand binds to a specific protein and thus, each protein can be labeled with a DNA strand with a specific sequence. DNA bound to the target protein can be analyzed by NGS to elucidate the ligands with high affinity. While it is complicated to analyze small molecules one by one, it has been realized simultaneous parallel analysis by encoding the ligand information into DNA sequence information.[13] By applying this technique, metabolite information can be encoded in DNA.

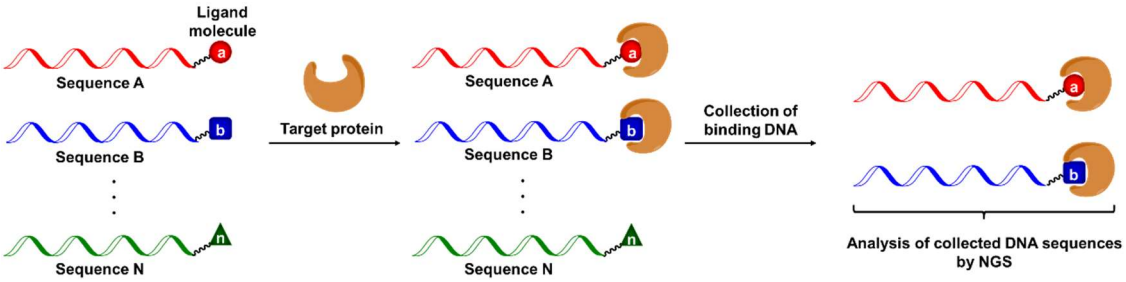

Figure 4. Schematic illustration of DNA encoded strategy.

Biotin-avidin interaction and its application

Avidin is a tetrameric protein and exist in chicken albumen. As shown in Figure 5, avidin protein bind with four biotin molecules tightly, and the K_d value of biotin-avidin interaction is estimated to be 10^{-15} mol/L.[14] This binding affinity approximately equals to covalent bond and is not responsive for any factor such as pH, solvent and denaturation agents. Because of this high affinity, biotin-avidin interaction is used for many biological materials to couple various biomaterials.[15]

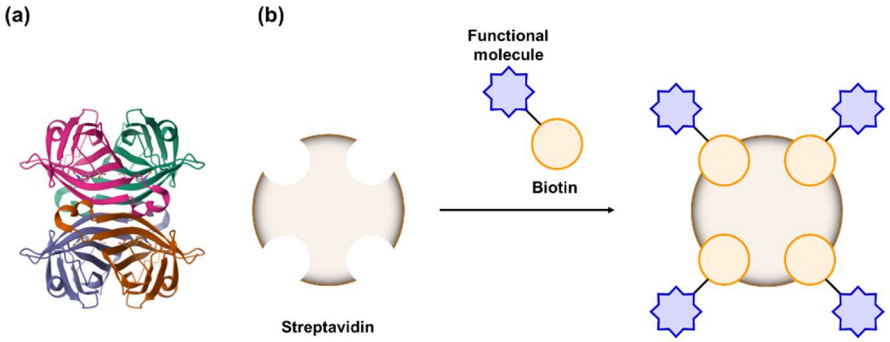

Figure 5. (a) Structure of streptavidin (by PDB: 1STP) (b) Illustration of biotin modified functional molecule and streptavidin conjugate.

For instance, the sandwich ELISA assay used biotin-streptavidin interaction (Figure 6). In the assay, a sample containing the target antigen was added to wells labeled with an antigen capturing antibody.[16] A biotin-labeled target recognition antibody was added, and then, streptavidin modified horseradish peroxidase (HRP) was added so that HRP is immobilized in the wells only in the presence of the target antigen. Finally, a substrate, absorbance of which changed during the enzymatic reaction of HRP was added, and the amount of target antigen could be quantified from the colorimetric change. The biotin molecule is small enough that large enzymes can quantitatively bind to the antibody and measure the target antigen. On the other hand, streptavidin was introduced into the surface of inorganic particles such as gold nanoparticle, magnetic beads and so on. They

were used for lateral flow immunoassay.[17] Shen and coworkers achieved detection of target antigen by using two types of gold nanoparticles. The one was modified by biotin and antibody, while the another was modified by streptavidin. Thus, target biomolecules such as protein were identified using biotinylated or streptavidin coated materials.[18]

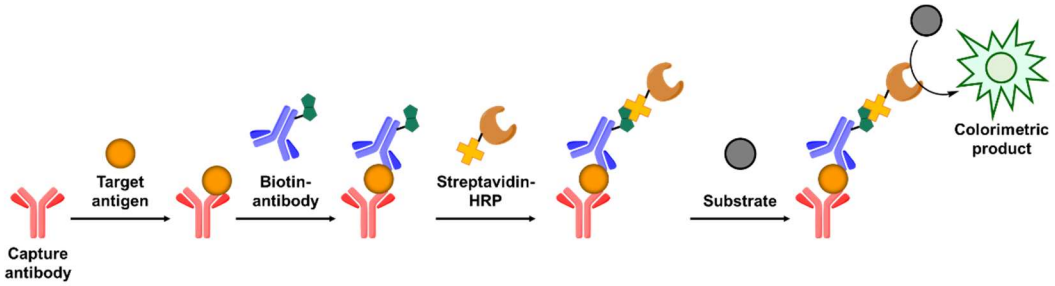

Figure 6. Schematic illustration of sandwich ELISA using biotin-streptavidin interaction.

Outline of this thesis

As mentioned above, functional artificial nucleic acids and groups of biofunctional molecules have many applications in the academic field of chemical biology. In this thesis, we developed a molecular system of metabolite analysis using these functional molecules. Out targets are glutathione (GSH), hypochlorite ion, lactate, glucose and hydrogen peroxide, all of which have important roles in living cells. Derived from these studies, the functional molecules have been also applied to the design of prodrugs, which showed smart medical activities without side effects.

In **Chapters 1-4**, we achieved the detection of metabolites and cell-derived small molecules by utilizing artificial nucleic acids. Specifically, it was shown in **Chapter 1** that it is possible to detect cell-derived glutathione by encoding its information into DNA sequence information. In **Chapter 2**, the system developed in **Chapter 1** was applied to the detection of hypochlorite ion. In **Chapter 3**, we succeeded in encoding the information of lactate and glucose, which are less reactive metabolites, into DNA

sequence information by using enzymatic reactions. In **Chapter 4**, by utilizing ethanol precipitation, the molecular system using solid phase analysis in **Chapters 1-3** was changed to a liquid phase analysis, and glutathione and hydrogen peroxide were successfully detected. For the detection of glutathione, we succeeded in improving the sensitivity by about 100-fold compared to the conventional systems. In **Chater 5**, a prodrug based on biotin-avidin interaction was developed. In this prodrug, a biotin modified photo-responsive prodrug and a biotin modified DNA aptamer were complexed with streptavidin as a scaffold. This prodrug showed cytotoxic effect under photoirradiation conditions against the cells which expressed the target antigen.

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Chapter 1

Encoding Thiol Metabolite Information into a DNA Sequence by Disulfide Exchange Reaction on Oligodeoxynucleotides for Parallel Analysis of the Metabolite and mRNA

Abstract

We designed a methodology to encode the thiol metabolite information into a DNA sequence. Thiol metabolites, such as glutathione (GSH) significantly contributes to regulating redox balance in the cells. We constructed a molecular system to quantify GSH by releasing DNA from magnetic beads upon the disulfide exchange reaction. We designed the molecular system as follows: for the conversion of thiol metabolite information into DNA sequence, we used a disulfide exchange reaction between the disulfide (reaction site) tethered oligonucleotide and thiol group of target metabolites. GSH and mRNA in the cell extract were successfully quantified from the samples obtained by DNA encoding and reverse transcription. The present protocol allowed parallel analysis of thiol metabolite and mRNA.

1-1. Introduction

Methodologies using DNA encoding have been used for designing molecular systems to screen the ligand[1] and detect biological targets.[2] DNA can be amplified, allowing high-sensitive detection of a small amount of their target molecule. Next-generation sequencing (NGS) can analyze vast DNA sequences, facilitating high throughput analysis. Furthermore, single-cell transcriptome analysis using the cell barcoding sequence has been paid attention.[3] The detection of multi-component target biomolecules with transcriptome has also been achieved by matching the target biomolecule with DNA sequence.[4]

In the field of medicine, metabolome analysis has been actively conducted in recent years, combining separation methods such as capillary electrophoresis (CE), liquid chromatography (LC), and gas chromatography (GC) with mass spectrometry techniques. Metabolome analysis has been conducted in the cohort study to discover the disease biomarker.[5] However, a technology for jointly analyzing the metabolome and transcriptome is lacking as the metabolites cannot be amplified by polymerase chain reaction (PCR), unlike DNA. Therefore, metabolome and transcriptome analyses must be performed separately, which is time-consuming and expensive for multi-omics analysis. In addition, owing to the procedure that separates the metabolites, the lack of throughput is a major issue. In this study, we attempted to convert the reactive metabolite information into DNA sequences using chemical reactions (Figure 1a). The proposed methodology can overcome the low throughput problem of conventional metabolome analysis and realize integrated analysis with the transcriptome and genome.

As a proof of concept, we employed thiol metabolites as reactive metabolites, especially glutathione (GSH), which is the most abundant thiol metabolite and significantly

contributes to regulating redox balance in the cells.[6–8] Recently, several DNA based probe have been developed mainly using the fluorescence change.[9] However, it is difficult to apply for the amplification strategy such as qPCR. In this study, we designed the molecular system using the functional beads as follows: for the conversion of thiol metabolite information into DNA sequence, we used a disulfide exchange reaction between the disulfide (reactive site) tethered oligonucleotide and thiol group of target metabolites (Figure 1b). We prepared functional beads on which double-stranded oligodeoxynucleotides (dsDNA) bearing disulfide bond were immobilized. The dsDNA comprises a reaction ODN that is selectively cleaved by thiol metabolite, mainly GSH in the sample and codes ODN, which has target specific sequence to indicate the thiol metabolites information (Figure 1c).

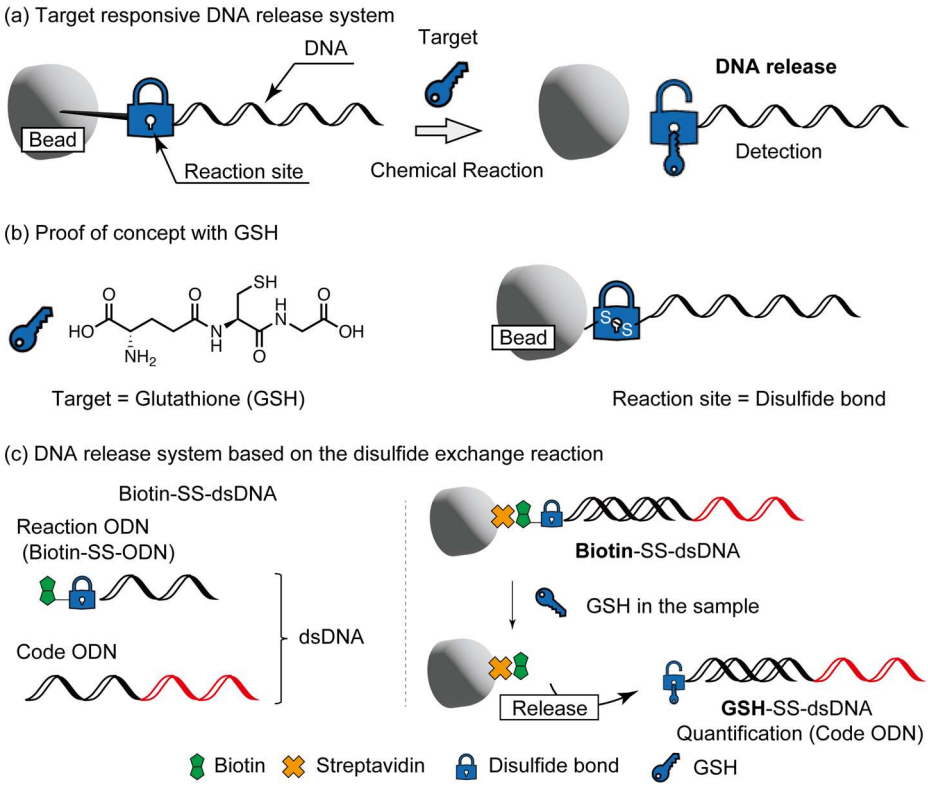

Figure 1. (a) Illustration of target responsive DNA release system using functional bead to encode reactive metabolite information into DNA sequence. (b) GSH as the representative thiol metabolite and magnetic beads labeled with disulfide (reaction site) tethered oligodeoxynucleotide (c) Functional dsDNA composing the reaction ODN and code ODN. The beads system using functional dsDNA, which releases the dsDNA upon the disulfide exchange reaction with the thiol metabolites, mainly GSH in the sample.

When the samples including GSH were added to the magnetic beads, GSH reacted with the disulfide bond of the reaction ODN (biotin-SS-ODN) to form adduct and release dsDNA (GSH-SS-dsDNA) from the magnetic beads. As the amount of released dsDNA was proportional to the GSH amount, the GSH concentration in the samples can be quantified from the released code ODN. For the parallel analysis, we also defined HO-1 mRNA as a target mRNA to monitor oxidative stress. HO-1 mRNA can be extracted and quantified using a portion of the sample. Eventually, GSH and HO-1 mRNA in the cell

extract were successfully quantified from the samples obtained by DNA encoding and reverse transcription.

1-2. Results and Discussion

Initially, we synthesized the reaction ODN having a disulfide bond and a biotin unit (biotin-SS-ODN) at 3'-end. The synthesis was achieved by the coupling of the biotin derivative with disulfide bond and amine-terminated ODN. We also prepared another ODN without a disulfide bond (biotin-ODN) as a reference ODN. These functional ODNs were purified by reversed-phase HPLC and identified by MALDI-TOF MS.

Subsequently, we evaluated the reaction of the biotin-SS-ODN with GSH by HPLC (Figure 2a). As shown in Figure 2b, biotin-SS-ODN reacted with GSH in phosphate buffer to give the GSH adduct ODN (GSH-SS-ODN), releasing biotin derivative bearing thiol unit. The GSH-SS-ODN was identified via MALDI-TOF MS measurement after collection by HPLC. The yield of GSH-SS-ODN increased in a dose-dependent manner. The conversion rate calculated from the peak areas of product ODN (GSH-SS-ODN) and reaction ODN (biotin-SS-ODN) in HPLC revealed that the reaction of biotin-SS-ODN with GSH proceeded quantitatively (Figure 2c).

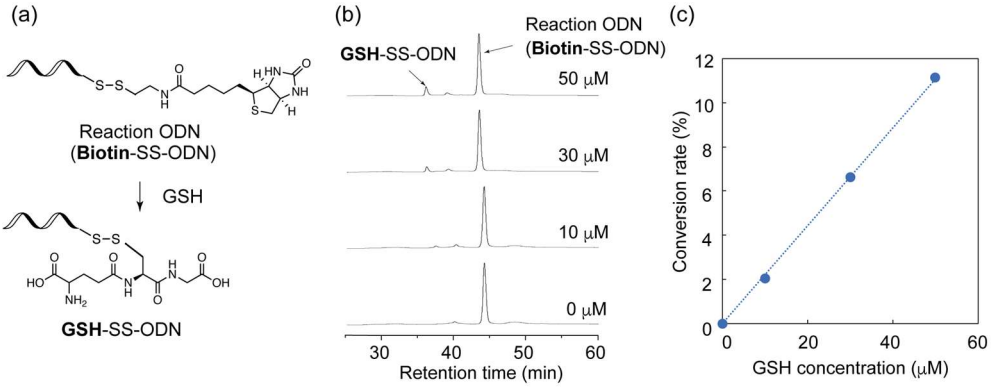

Figure 2. (a) Cleavage reaction of reaction ODN (biotin-SS-ODN) with GSH. (b) Reaction monitoring using HPLC. Briefly, 10 mM reaction ODN was reacted with 0, 10, 30, and 50 mM GSH in the 250 mM phosphate buffer (pH 8.4) for 16 h. (c) Conversion rate (GSH-SS-ODN / (Biotin-SS-ODN + GSH-SS-ODN)) was determined using each peak area.

We also evaluated the disulfide bond cleavage on biotin-SS-ODN, which was located on streptavidin (Figure 3). Duplex DNA comprising biotin-SS-ODN and 60-mer code ODN was formed (biotin-SS-dsDNA), and it was then bound with streptavidin to give a streptavidin–SS-dsDNA complex. The reaction was monitored by native PAGE and DNA staining. When GSH was added to the streptavidin–SS–dsDNA complex, the bands derived from the complex decreased; however, the duplex DNA (GSH-SS-dsDNA), which was released from the complex owing to the addition of GSH, appeared. In a separate experiment, we prepared a similar complex, which did not have a disulfide bond (streptavidin–dsDNA complex) as a reference complex. The addition of GSH to the streptavidin–dsDNA complex resulted in no reaction, indicating that the disulfide bond is a key motif for releasing dsDNA from the complex.

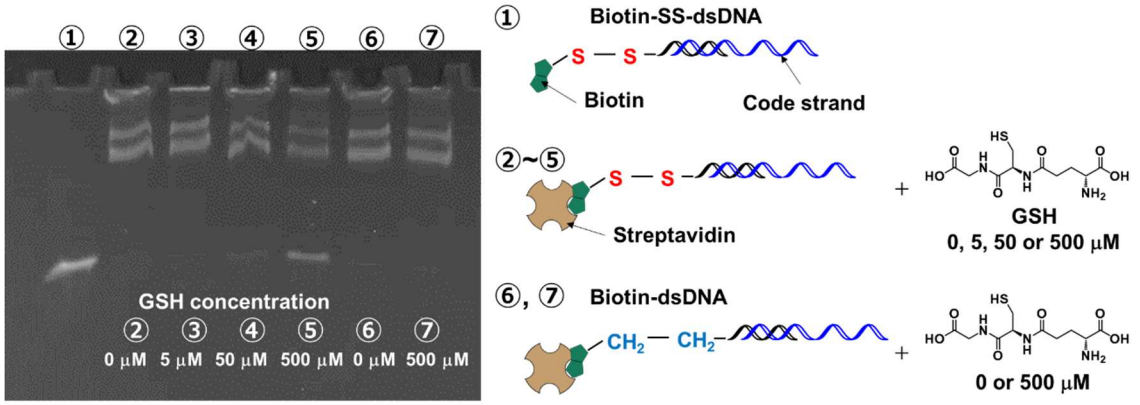

Figure 3 Native PAGE for monitoring of dsDNA release. 10% native polyacrylamide gel electrophoresis (15 mA, 100 min). Lane 1: 5 pmol biotin-SS-dsDNA. Lane 2–5: 5 pmol biotin-SS-dsDNA + 10 pmol streptavidin incubated for 14 h at room temperature with GSH (0, 0.1, 1 or 10 nmol, 20 μL). Lane 6, 7: 5 pmol biotin-dsDNA + 10 pmol streptavidin incubated for 14 h at room temperature with GSH (0 or 10 nmol, 20 μL).

Having the suitable complex for the quantification of GSH in hand, we then tested the release of GSH-SS-dsDNA from streptavidin-coated magnetic beads by the addition of GSH and quantification of released ODNs (Figure 4a).

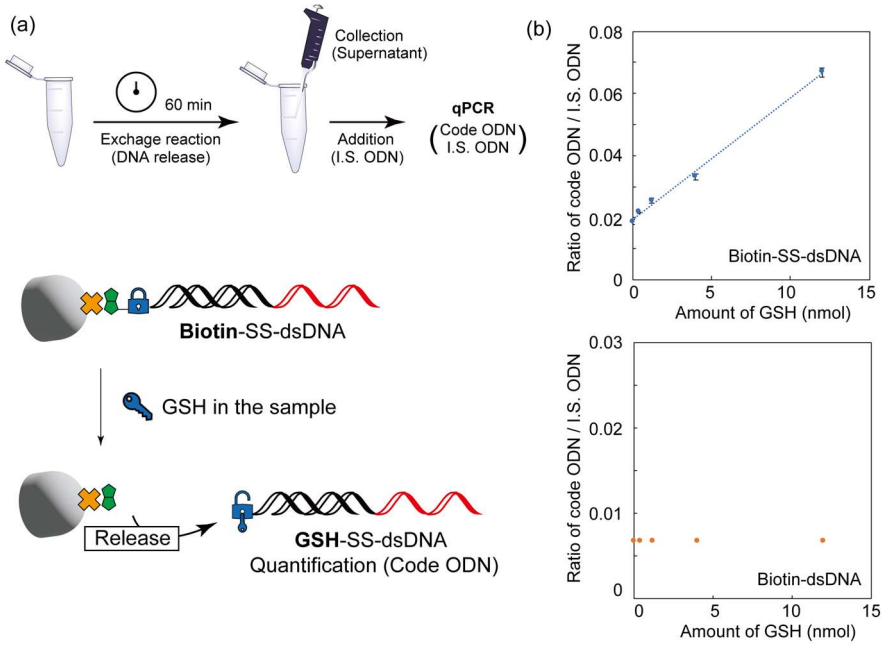

Figure 4. (a) Procedure to quantify the amount of GSH in the sample using the released dsDNA and I.S. ODN. (b) Dose dependency of released dsDNA when biotin-SS-dsDNA (Data = mean ± SD, n = 3) or biotin-dsDNA (n = 1) was loaded on streptavidin-coated magnetic beads.

The amount of released ODNs (code ODN) was quantified by qPCR using single-stranded ODN with a different sequence as an internal standard. As shown in Figure 4b, GSH-SS-dsDNA was released from the beads by the addition of GSH, and the qPCR revealed that the amount of GSH-SS-dsDNA was proportional to the amount of GSH added. However, when double-stranded DNA without disulfide bond (biotin-dsDNA) was used in place of biotin-SS-dsDNA, dsDNA was negligibly detected. Thus, the release of GSH-SS-dsDNA from magnetic beads was triggered by the cleavage of the disulfide bond. A similar evaluation was performed for cysteine (Cys), the second most abundant thiol metabolite after GSH in the cell, suggesting a two-fold faster reaction rate for Cys than for GSH (Figure 5). This difference in reaction rate may be due to the more negative charge of GSH than that of Cys, which inhibited the reaction owing to electrostatic

repulsion. These reaction properties of Cys make the reaction cumbersome; however, the amount of Cys present in the cell is very low compared to that of GSH.[10]

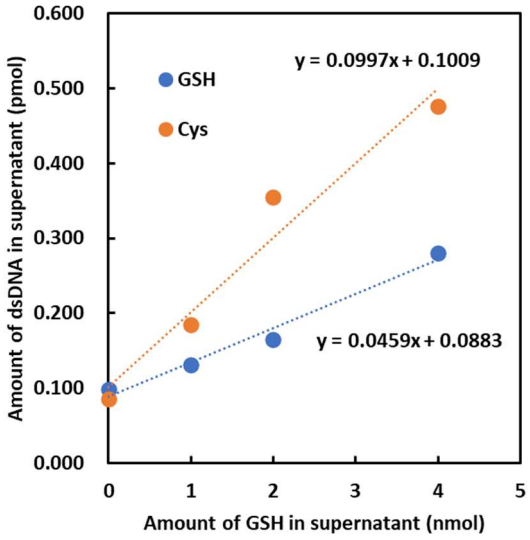

Figure 5. dsDNA release from magnetic beads upon the GSH or cysteine reaction. DNA release upon the reaction between the biotin-SS-labeled magnetic beads and GSH or cysteine (0, 1, 2 or 4 nmol, 1 h incubation).

Therefore, when this system is applied to cell extract, selectivity for GSH can be ensured. We also confirmed an increase in the amount of GSH-SS-dsDNA released upon reduction by GSH, which is related to the amount of biotin-SS-dsDNA loaded on the beads (appropriate amount is 5 pmol, Figure 6). In addition, we found that the appropriate pH condition for this reaction was pH 8.6–8.9 (Figure 7).

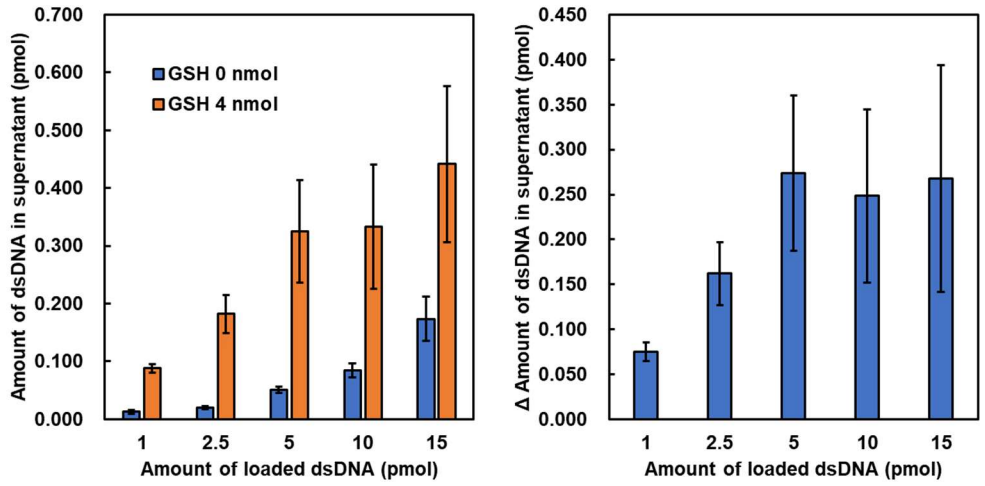

Figure 6. Evaluation of release dsDNA depending on the loaded dsDNA amount. dsDNA release from biotin-SS-dsDNA labeled magnetic beads (amount of loaded dsDNA 1, 2.5, 5, 10, 15 pmol) upon the reaction with GSH (0 or 4 nmol) (Δ amount of dsDNA in supernatant = amount of release dsDNA from beads incubated with GSH – without GSH, Data = mean ± SE, three independent experiments)

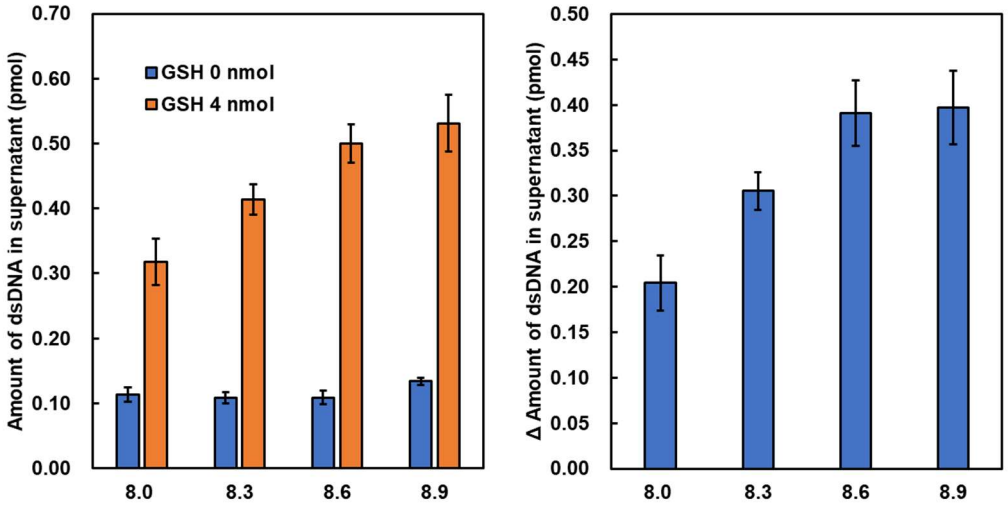

Figure 7. Evaluation of release dsDNA depending on the pH conditions. dsDNA release from magnetic beads upon the reaction with GSH (0, 4 nmol) under several pH condition (pH 8.0, 8.3, 8.6, 8.9). (Δ amount of dsDNA in supernatant = amount of release dsDNA from beads incubated with GSH – without GSH, Data = mean ± SE, three independent experiments)

Further attempts were made to monitor the change in GSH amount by the present system during oxidative stress induced by hydrogen peroxide exposure. We also monitored the mRNA level of the oxidative stress marker (HO-1). Briefly, a joint analysis of metabolite and mRNA was attempted. We selected A549 cells, a lung cancer cell line, to monitor the GSH change because GSH levels in A549 cells are relatively high among cancer cell lines.[11] The intracellular GSH amount decreased rapidly upon exposure to hydrogen peroxide, whereas the HO-1 levels increased.

We compared their levels with or without hydrogen peroxide treatment. Figure 8a shows the protocols for the measurement of GSH and HO-1 mRNA levels in A549 cells. The cell extract was harvested after treatment with or without hydrogen peroxide, and the cell extract was then added to the beads prepared in this study to convert GSH information into DNA sequence (code ODN), which were subjected to qPCR for GSH quantification. Simultaneously, a portion of cell extract was also subjected to RT-qPCR for HO-1 mRNA quantification. As shown in Figure 8b, c, after exposure to hydrogen peroxide for 12 h, qPCR experiments revealed that the GSH level significantly decreased, whereas the HO-1 mRNA level increased.[12] Thus, hydrogen peroxide treatment caused oxidative stress and altered intracellular mRNA (HO-1) and metabolite (GSH) levels. In a separate experiment, the colorimetric quantification of GSH in the same sample was conducted using a commercially available kit. The same level of GSH was observed in the sample, indicating that the GSH level in a cell extract can be quantified by a simple operation of the present protocol. Thus, this protocol allowed parallel analysis of metabolite and mRNA by DNA encoding and reverse transcription.

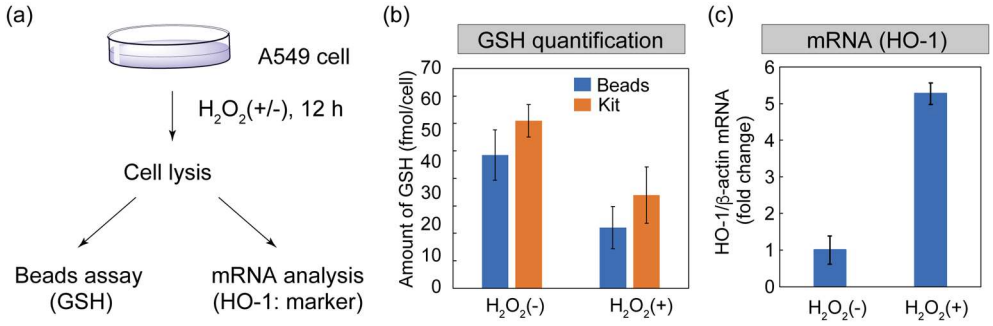

Figure 8. (a) Procedure to monitor the GSH and mRNA expression level (HO-1) in cell extract. A549 cells were treated with the medium containing 0 or 182 mM of hydrogen peroxide for 12 h. (b) Amount of GSH per cell, which was quantified using the functional beads system and a commercial quantitative kit. (c) Expression level of HO-1 under 0 or 182 mM H_2O_2 exposure condition. Data = mean ± SD (n = 3).

This strategy can encode the thiol metabolite information into DNA sequence only mixing the functional magnetic beads and the sample solution. Although the range to quantify the GSH in this method was almost similar to other GSH measurement assays,[13] this strategy can collect released DNA from beads with mRNA by precipitation technique. Therefore, this feature prompted us to establish the seamless DNA encoding and reverse transcription (Figure 9). During 1 h incubation with the cell extract, the DNA was released from the magnetic beads. Then the sample was performed the precipitation technique to collect the released DNA and mRNA simultaneously. The reverse transcription step was performed sequentially to give the cDNA. In fact, we succeeded in the detection of thiol metabolites. The amount of released DNA was suppressed under the condition with *N*-methyl-maleimide (NMM), which react with the thiol group of metabolites via the Michael reaction. In addition, we confirmed the presence of β-actin mRNA precisely.

These data suggested that the thiol metabolite and mRNA detection was achieved through the seamless DNA encoding and reverse transcription.

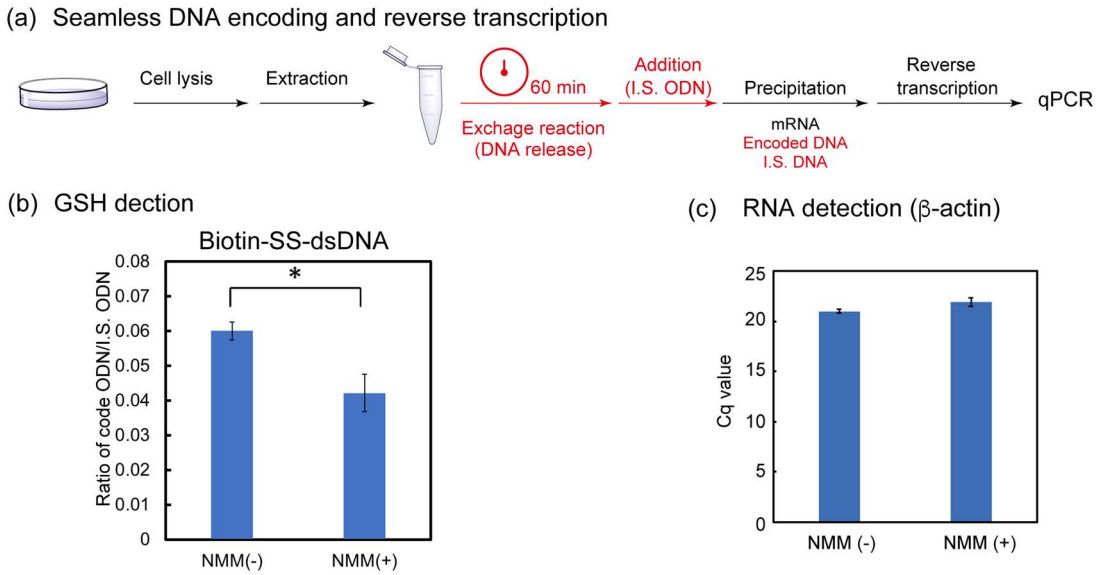

Figure 9. Seamless DNA encoding and reverse transcription. (a) Procedure of DNA encoding and reverse transcription. We inserted the steps to encode the thiol metabolite information in the cell extract before the RT-qPCR and collected the encoded DNA and I.S.DNA using the precipitation technique, which was described in red. (b) GSH detection in the cell extract with or without additional NMM treatment. (c) Cq value of β-acitin cDNA detection. Data = means ± SD (n = 3, * indicates $p<0.1$)

1-3 Conclusion

In conclusion, we demonstrated that the thiol metabolites information could be converted into DNA sequence, thereby achieving the joint analysis of the metabolite and mRNA. The present methodology for the quantification of thiol metabolite, mainly GSH is quite simple: a sample (cell extract) was added to magnetic beads loaded with functional dsDNA, and supernatant was then collected after 1 h incubation. On the beads, a disulfide

bond exchange reaction occurred in response to the metabolite GSH, releasing GSH-SS-dsDNA. Based on this method, GSH information was converted to DNA sequence (code ODN). Thus, after DNA encoding and reverse transcription, the target metabolite and transcriptome could be analyzed in parallel by using NGS technology. In addition, this strategy has possibility to apply other class of reactive molecules. Thus, this DNA encoding strategy could be applied to the multiple analysis of reactive biological molecules. Further work is now underway in our laboratory to expand the target molecules.

1-4 Experimental section

General Methods

Reagents were purchased from Wako pure chemical industries, Sigma Aldrich, Nacalai Tesque, Takara Bio, Dojindo, Tokyo chemical industries, New England BioLabs, Corning, Promega, Beckman Coulter Inc., and eurofins Genomics. Analytical and preparative high-performance liquid chromatographies were performed on a ChromAssist Data Station (Pump Chromaster® 5110, UV detector Chromaster® 5410, HITACHI) with reversed phase column (InerrtsilvODS-3, GL Science Inc., ϕ 1.0 mm×250 mm (particle size; 5 μm), ϕ 4.6 mm×250 mm (particle size; 5 μm)). The column eluents were monitored by the UV absorbance at 260 nm at a flow rate of 0.6 (analysis) $mLmin^{-1}$ with

a 0–30% (over 60 min, Figure 2b) or 10–35% (over 30 min, Figure S1) gradient of acetonitrile (can) / triethylammonium acetate buffer (TEAA buffer) (100 mM, pH 7.0) or 3.0 (preparative) $mLmin^{-1}$ with a 10–35% (over 30 min, for synthesis) gradient of ACN/TEAA buffer (100 mM, pH 7.0). MALDI-TOF mass spectrometry were recorded on an AXIMER-LNR spectrometer (SHIMADZU). DNA hybridization was carried out in iCycler™ Thermal Cycler (Bio Rad). Native polyacrylamide gel electrophoresis was carried out using an AE-8800 COSTAPOWER 3000 (ATTO). The gel image was acquired by FluoroPhoreStar 3000 (LED light irradiation 530 nm) (anatech). Quantitative PCR (qPCR) analysis was carried out with MyGo Mini S Real Time PCR (IT-IS Life Science Ltd.). TNB absorbance was recorded on a xMarkTM microplate absorbance spectrophotometer (BIO RAD). Quantitative analysis of DNA or RNA was carried out with Quantus™ Fluorometer (Promega). A549 was given from Dr. Aoi Son (Kyoto University). DNA sequences are shown in Table 1.

Table 1. Sequences in this study

Strand name	Sequences (5' to 3')
Reaction ODN	GGT CCA ATC TAC AGG AAT TC-NH_2
Code ODN	GAA TTC CTG TAG ATT GGA CCT TTT TTT TTT TTT TTT TTT CGA CCC TAA GCA TAC ATA CCT
Forward primer	AGG TAT GTA TGC TTA GGG TCG
Reverse primer	GAA TTC CTG TAG ATT GGA CC
Internal standard ODN	CCA GGT TAG ATG TCC TTA AGT TTT TTT TTT TTT TTT TTT TCC ATA CAT ACG AAT CCC AGC
Forward primer (for I.S. ODN)	GCT GGG ATT CGT ATG TAT GGA
Reverse primer (for I.S. ODN)	CCA GGT TAG ATG TCC TTA AG
Forward primer of HO-1	CAG GCA GAG AAT GCT GAT TC
Reverse primer of HO-1	GCT CTT CTG GGA AGT AGA CAG G
Forward primer of β-actin	GAT CAT TGC TCC TCC TGA GC
Reverse primer of β-actin	ACT CCG CTT GCT GAT CCA C

Synthesis

Synthesis of Biotin-SS-ODN

20 μL of carbonate-bicarbonate buffer (750 mM pH 8.7) containing reaction oligodeoxynucleotide (ODN) (5.0 nmol) was added to 130 μL of Biotin-SS-Sulfo-OSu (Dojindo) (2.1 μmol) in milliQ water. The resulting solution was kept at room temperature for overnight and was purified using Micro Bio-Spin™ 6 Columns (Bio-Rad) and milliQ water as an elution solution. Moreover, the sample was purified by HPLC using ODS-3 column (ϕ 4.6 mm×250 mm (particle size; 5 μm)). The concentration of the oligomer was determined by complete digestion with alkaline phosphatase (AP), nuclease P1 (PI) and phosphodiesterase I at 37°C for overnight. We monitored the absorbance from the digested dA, dT, dG, dC and calculated the concentration using calibration curve of each deoxyribonucleoside to determine the concentration of synthesized oligomer. Identities of synthesized oligomers were confirmed by MALDI-TOF MS spectrometry ($[M-H]^-$calcd. 6695.2, found 6694.9).

Synthesis of Biotin-ODN

17 μL of carbonate-bicarbonate buffer (706 mM pH 8.7) containing reaction ODN (5.0 nmol) was added to 100 μL of Biotin-AC_5 Sulfo-OSu (Dojindo) (2.2 μmol) in milliQ

water. The resulting solution was kept at room temperature for overnight and was purified using Micro Bio-Spin™ 6 Columns (Bio-Rad) and milliQ water as an elution solution. Moreover, the sample was purified by HPLC using ODS-3 column (ϕ 4.6 mm×250 mm (particle size; 5 μm)). The concentration of the oligomer was determined by complete digestion with AP, PI and phosphodiesterase I at 37°C for overnight. We monitored the absorbance from the digested dA, dT, dG, dC and calculated the concentration using calibration curve of each deoxyribonucleoside to determine the concentration of synthesized oligomer. Identities of synthesized oligomers were confirmed by MALDI-TOF MS spectrometry ($[M-H]^-$calcd. 6645.3, found 6645.4).

Reaction monitoring of biotin-SS-ODN

Reaction monitoring of reaction ODN (biotin-SS-ODN) and GSH in the solution

10 μM biotin-SS-ODN was reacted with 0, 10, 30 or 50 μM GSH in PB (250 mM, pH 8.4) for 16 h at room temperature. The reaction mixture was analyzed by HPLC.

DNA hybridization

2 μM code ODN and reaction ODN (biotin-SS-ODN) or biotin-ODN were hybridized in milliQ water containing 5 mM $MgCl_2$. DNA was heated to 95°C for 5 min and then cooled at a rate of 0.1°C per sec to 25°C.

Reaction monitoring between biotin-SS-dsDNA and GSH on streptavidin using native PAGE

1 μL of streptavidin (10 pmol) in PBS was added to 17 μL phosphate buffer (294 mM, pH 8.4) containing biotin-SS-dsDNA or biotin-dsDNA (5 pmol), and incubated at room temperature for 30 min. After incubation, the solution was added to 2 μL of GSH (0, 0.1, 1 or 10 nmol) in milliQ water, and incubated room temperature for 14 h. Each solution was analyzed by 10% native PAGE. DNA was stained by Gel Red.

Binding of biotin-SS-dsDNA with streptavidin coated magnetic beads

Biotin-SS-dsDNA or biotin-dsDNA was bound with streptavidin coated magnetic beads (50 μg, Magnosphere^{TM} MS300/Streptavidin, JSR corporation) according to the manufacturer's procedure as follows. Biotin-SS-dsDNA was incubated in the binding buffer (10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 1 M NaCl, 0.05% Tween20)

containing streptavidin magnetic beads for 10 min. Then, the beads were washed with binding buffer twice and suspended with the binding buffer (20 μL/sample).

Evaluation of disulfide bond dependent reaction with GSH on magnetic beads

Biotin-SS-dsDNA or biotin-dsDNA (5 pmol) labeled magnetic beads (50 μg) were washed with 250 mM PB (pH 8.6, 20 μL). Then, GSH (0, 0.4, 1.2, 4 or 12 nmol) in 250 mM PB (pH 8.6, 20 μL) was mixed with magnetic beads and incubated for 1 h at room temperature by gentle shaking. The supernatant (10 μL) was collected using magnet, and internal standard ODN was added (5 pmol, 10 μL). After dilution using milliQ water (×10,000), the sample (the amount of code ODN and I.S. ODN) was quantified using qPCR to obtain the ratio. qPCR was performed according to the manufacturer's procedure using Luna Universal qPCR Master Mix (NEW ENGLAND Bio Labs): 2 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C.

dsDNA release from magnetic beads upon the GSH or cysteine reaction

Biotin-SS-dsDNA (5 pmol) labeled magnetic beads (50 μg) were washed with 250 mM PB (pH 8.6, 20 μL). Then GSH (0, 1, 2 or 4 nmol) or Cys (0, 1, 2 or 4 nmol) in 250 mM PB (pH 8.6, 20 μL) was mixed with magnetic beads and incubated for 1 h at room

temperature by gentle shaking. The supernatant (10 μL) was diluted using milliQ water (×10,000) and quantified using qPCR as described above.

Evaluation of release dsDNA depending on the loaded dsDNA amount

Biotin-SS-dsDNA (1, 2.5, 5, 10 or 15 pmol) was bound with streptavidin coated magnetic beads (50 μg) describe above. Biotin-SS-dsDNA labeled magnetic beads were washed with 250 mM PB (pH 8.6, 10 μL). Then GSH (0 or 4 nmol) in 250 mM PB (pH 8.6, 20 μL) was mixed with magnetic beads and incubated for 1 h at room temperature by gentle shaking. The supernatant (10 μL) was diluted using milliQ water (×10,000) and quantified with qPCR as described above.

Evaluation of release dsDNA depending on the pH conditions

Biotin-SS-dsDNA (5 pmol) labeled magnetic beads (50 μg) were washed with 250 mM PB (pH 8.0, 8.3, 8.6 or 8.9, 10 μL). Then GSH (0 or 4 nmol) in 250 mM PB (pH 8.0, 8.3, 8.6 or 8.9, 20 μL) was mixed with magnetic beads and incubated for 1 h at room temperature by gentle shaking. The supernatant (10 μL) was diluted using milliQ water (×10,000) and quantified with qPCR as described above.

Quantification of intracellular GSH and mRNA

Cell culture

Human lung cancer cell line, A549 and human cervix epithelial adenocarcinoma, HeLa was cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin. The cells were maintained at 37°C in 5% CO_2 / 95% air and were kept in a logarithmic growth phase by routine passages every 2−3 days. Prior to the use of cells, the densities of cells were determined using a hemocytometer.

Preparation of cell extract sample for quantification of GSH and HO-1 mRNA

A549 cells (3.0×10^5 cells per well) were seeded onto 6 well plate in 3 mL DMEM. Plate was maintained at 37°C in 5% CO_2 / 95% air incubator for 24 h. 300 μL of 0 or 2 mM H_2O_2 (f.c. 0 or 182 μM H_2O_2) were added into each well and incubated for 12 h at 37°C in 5% CO_2 / 95% air incubator. After washing with D-PBS (-) twice, lysis buffer (10 mM Tris-HCl (pH7.4), 2 mM EDTA, 0.05% Triton-X) was added into each well and incubated for 15 min on ice. Then, cell lysates (500 μL) were collected and extracted with 200 μL of $CHCl_3$. After centrifugation at 12,000 g for 15 min at 4°C, aqueous layer was used for GSH quantification (90 μL) and HO-1 mRNA quantification (200 μL).

RNA quantification (RT-qPCR)

8 M LiCl aq. (20 μL) and isopropanol (220 μL) were mixed with each cell extract sample (200 μL) and incubated for 10 min at room temperature. After centrifugation at 12,000 g for 5 min at 4°C, supernatant was removed. 500 μL of 75% EtOH was added to the pellet and centrifuged at 12,000 g for 5 min at 4°C. Then, supernatant was removed, and the pellet was dried in air for 30 min. The pellet was solved with 10 μL of DEPC treated water (Nippon Gene) and quantified RNA concentration by Quantus™ Fluorometer using QuantiFluor® RNA system (Promega). After dilution of RNA in the same concentration, RNA was reverse-transcribed into cDNA using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo) according to the manufacturer's instructions. Quantification was conducted using Luna Universal qPCR Master Mix (New England Biolabs). The PCR reaction conditions were as follows: initial denaturation 2 min at 95°C, followed by 45 amplification cycles of 10 sec at 95°C, 30 sec at 60°C. mRNA of β-actin was used as an internal reference gene. Relative values for mRNA levels were calculated using the following formula: fold change = $2^{-\Delta\Delta Cq}$.

GSH quantification using the functional magnetic beads

90 μL of cell extract samples were lyophilized and added 40 μL of 250 mM PB (pH8.6). Biotin-SS-dsDNA labeled magnetic beads (50 μg) were washed with 250 mM PB (pH 8.6, 50 μL). 20 μL of cell extract solution in 250 mM PB (pH 8.6) was added to the biotin-SS-dsDNA labeled magnetic beads (50 μg) and incubated for 1 h at room temperature by gentle shaking. The supernatant (10 μL) was collected using magnet, and internal standard ODN was added (5 pmol, 10 μL). After dilution using milliQ water (×10,000), the sample was quantified using qPCR as described above.

For GSH quantification: Lysate buffer (500 μL) were extracted with 200 μL of $CHCl_3$. After centrifugation at 12,000 g for 15 min at 4°C, aqueous layer was used for GSH calibration curve (90 μL). 90 μL of the sample were lyophilized and added 40 μL of GSH solution (0, 8 or 24 nmol) in 250 mM PB (pH8.6). Biotin-SS-dsDNA labeled magnetic beads (50 μg) were washed with 250 mM PB (pH 8.6, 50 μL). 20 μL of GSH calibration solution in 250 mM PB (pH 8.6) was added to the biotin-SS-dsDNA labeled magnetic beads (50 μg) and incubated for 1 h at room temperature by gentle shaking. The DNA quantification was performed as described above.

GSH quantification using colorimetry assay (Dojindo, GSSG/GSH Quantification Kit)

GSH quantification was performed according to the manufacturer's instructions with slight modification. Briefly, 90 μL of cell extract was lyophilized and added 80 μL of 0.5% 5-sulfo salicylic acid (SSA) solution. Then, 60 μL buffer solution was added to 40 μL of cell extract solution containing 0.5% SSA and incubated for 1 h at 37°C, and then 60 μL of DTNB in buffer solution was added to each well on 96 well plate. Each sample solution was incubated for 10 min at 37°C, and absorbance at 412 nm was recorded on a microplate absorbance spectrophotometer (xMarkTM microplate absorbance spectrophotometer).

Seamless DNA encoding and reverse transcription

Preparation of cell extract

HeLa cells (4.0×10^5 cells per well) were seeded onto 6 well plate in 3 mL DMEM. Plate was maintained at 37°C in 5% CO_2 / 95% air incubator for 24 h. After washing with D-PBS (-) twice, lysis buffer (10 mM Tris-HCl (pH7.4), 2 mM EDTA, 0.05% Triton-X) was added into each well and incubated for 15 min on ice. Then, cell lysates (500 μL) were collected and extracted with 200 μL of $CHCl_3$. After centrifugation at 12,000 g for

15 min at 4°C, aqueous layer (200 μL) was used for seamless quantification of RNA and GSH.

DNA encoding and reverse transcription

200 μL of cell extract solution was mixed with 0 or 100 mM N-methylmaleimide (NMM, 20 μL) and incubated for 30 min. Biotin-SS-dsDNA labeled magnetic beads (50 μg) were washed with lysis buffer (50 μL). Then, cell extract solution (200 μL) was added to the biotin-SS-dsDNA labeled magnetic beads and incubated for 1 h at room temperature by gentle shaking. The supernatant (200 μL) was collected using magnet. Then, 500 nM internal standard ODN (10 μL, 5 pmol), 8 M LiCl aq. (20 μL) and isopropanol (230 μL) were mixed with each reaction solution and incubated for 10 min at room temperature. After centrifugation at 12,000 g for 5 min at 4°C, supernatant was removed. 500 μL of 75% EtOH was added to the pellet and centrifuged at 12,000 g for 5 min at 4°C. Then, supernatant was removed, and the pellet was dried in air for 30 min. The pellet was diluted with 10 μL of DEPC treated water and quantified RNA concentration by Quantus Fluorometer using QuantiFluor RNA system (Promega). After dilution of RNA in the same concentration, RNA was reverse-transcribed into cDNA using the ReverTra Ace®

qPCR RT Master Mix with gDNA Remover (Toyobo) according to the manufacturer's instructions. Finally, quantification of the DNA was performed as described above.

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Chapter 2

Quantification of hypochlorite in cell lysates using phosphorothioate modified oligodeoxynucleotides.

Abstract

Hypochlorite ion is the one of the most important molecules in living cells. Conventionally, the quantification of hypochlorite ion was achieved by using fluorescent probes, however, they could not be used for the integrated analysis of genome and transcriptome. To overcome these limitations, we decided to use functional oligodeoxynucleotides (ODNs) to quantify hypochlorite ion. In this study, we designed molecular system for quantification of hypochlorite ion using the functional magnetic beads labeled by phosphorothioate (PS)-modified ODNs. We successfully quantified the hypochlorite ion in cell lysate using the system.

2-1 Introduction

Reactive oxygen species (ROS) is associated with a variety of physiological process in living cells.[1] In particular, hypochlorite ion is the one of ROS and have important roles in living cells. For example, hypochlorite ion generated by enzymatic reaction of myeloperoxidase (MPO) in neutrophil cells attack exogenous virus or bacteria.[2] Also, a level of hypochlorite ion is associated with various diseases.[3] Therefore, detection of hypochlorite ion in cells is important means for disease diagnosis and elucidation of diseases. Conventionally, some fluorescent probes were developed to visualize hypochlorite ions in living cells.[4,5,6] Nagano and coworkers reported silicon rhodamine-based fluorescent probes which have thiol group at 2' position.[4] These probes were oxidized by hypochlorite ions and showed high fluorescence. Jiang and coworkers reported naphthalene based probes which have *N,N*-dimethyl thiocarbamate group as a quencher. This probe reacted with hypochlorite ions and showed fluorescence by deprotection of the quencher.[5] These conventional probes are useful, however, they are difficult to use for integrated analysis with other analytical techniques such as genome, transcriptome and metabolome. Therefore, we decided to encode the information of hypochlorite ion into DNA sequences. In recent years, DNA-encoded technique has become capable of retaining a variety of molecular information such as proteins and small molecules, making it one of the most useful methodologies for analysis.[7] Previously, we reported methodology for detecting of intracellular glutathione (GSH) using functional ODNs modified by biotin via disulfide bond. We succeeded in quantification of intracellular GSH and mRNA simultaneously using magnetic beads labeled with functional ODNs.[8] Our molecular system achieved an integral analysis of transcriptome and metabolome. Therefore, these previous research contexts prompted us to apply this

methodology to detection of intracellular highly reactive small molecule for expansion of DNA-encoding strategy.

Herein, we designed the molecular system for detecting hypochlorite ions with magnetic beads labeled with functional ODNs which reacted with hypochlorite ion. These functional beads could release the DNA after reaction with hypochlorite ions. The amount of hypochlorite ions was quantified by quantification of released DNA from the magnetic beads. Eventually, using this system, we successfully quantified hypochlorite ion in cell lysate.

2-2 Result & Discussion

In this study, we aimed to develop molecular system for quantifying hypochlorite ion using functional magnetic beads labeled with phosphorothioate (PS) modified ODNs. PS-modified ODNs were known to be cleaved by reaction with hypochlorite ion.[9] We prepared PS-modified ODNs with biotin-modification at 5'-end (Biotin-PS-DNA) and functional magnetic beads which was labeled with duplex DNA (Biotin-PS-dsDNA) consisted of Biotin-PS-DNA and code DNA that encoded the information of an amount of hypochlorite ions. Biotin-PS-dsDNA was designed to be cleaved by reaction with hypochlorite ions and to be dissociated. As a result of dissociation of Biotin-PS-dsDNA, the code DNA would be released in the supernatant. Therefore, detection and quantification of hypochlorite ions were achieved by quantifying released code DNA after reaction with hypochlorite ions (Figure 1).

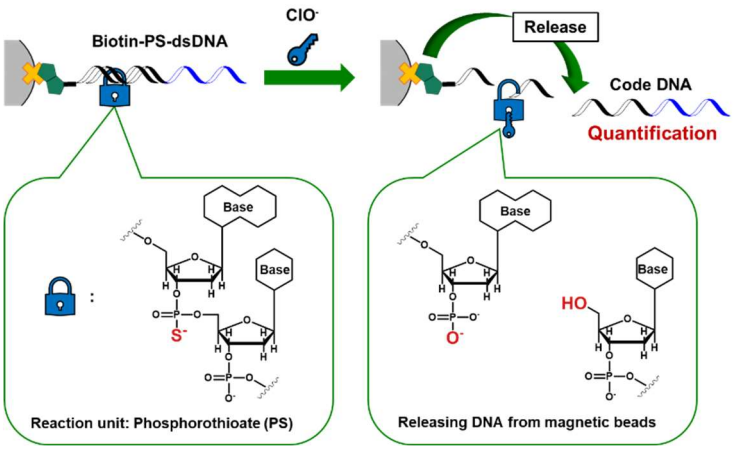

Figure 1. Schematic illustration of quantification of hypochlorite using functional magnetic beads labeled PS modified DNA.

First, we evaluated strand cleavage and dissociation of Biotin-PS-dsDNA by addition of hypochlorite ion. Fluorophore (FAM)-labeled biotin-PS-dsDNA (FAM-biotin-PS-dsDNA) was reacted with various concentration of sodium hypochlorite and dissociation of the duplex was evaluated by polyacrylamide electrophoresis (Figure 2a). In the reaction, FAM-biotin-PS-dsDNA (1 μM) was treated with 0-5 μM sodium hypochlorite in aqueous solution. As a result, Biotin-PS-dsDNA was disappeared and new band, which was attributed to shorter single stranded DNA (cleaved DNA), was appeared in dose-dependent manner (Figure 2c). The conversion rate was reached 29% when the ODNs reacted with 5 μM NaClO (Figure 2d). On the other hand, the control duplex without PS modification (Biotin-dsDNA) did not form ssDNA even after treatment with NaClO (Figure 2b). These results indicate that Biotin-PS-dsDNA could release code DNA by the reaction at PS group on ODNs with hypochlorite ions.

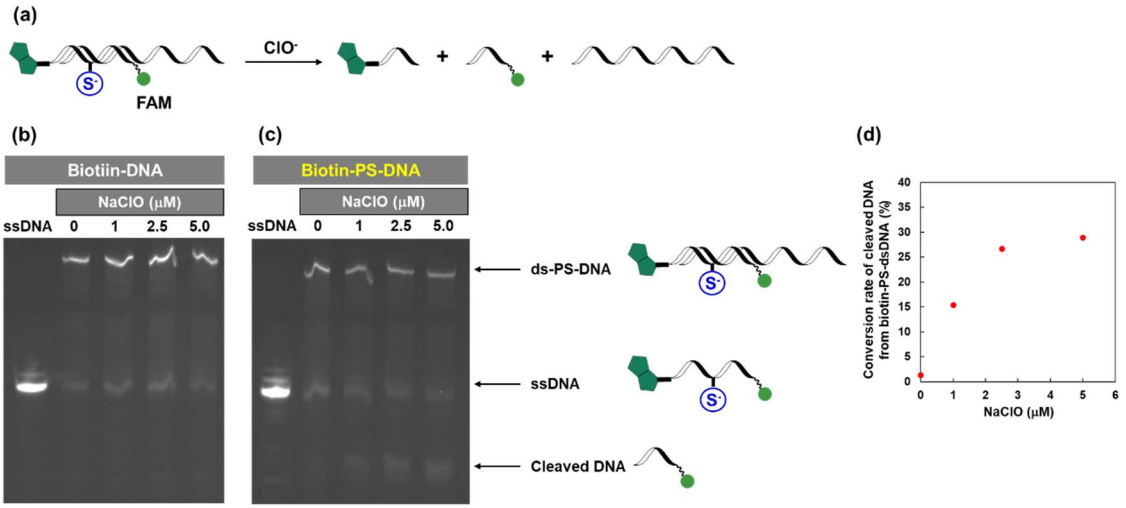

Figure 2. Native-PAGE analysis of 1μM dsDNA reacted with 0, 1, 2.5, 5.0 mM NaClO in PBS for 1 h at 37°C (a. Biotin-dsDNA, b. biotin-PS-dsDNA). (c) Conversion rate of the dsDNA to short DNA.

Next, we loaded Biotin-PS-dsDNA onto magnetic beads and evaluated whether code DNA was released into the supernatant upon addition of hypochlorite ions. 0-200 pmol of sodium hypochlorite was added to magnetic beads loaded with 5 pmol biotin-PS-dsDNA (Figure 3a). In this experiment, an internal standard DNA (I.S. DNA, 5 pmol) was mixed with the reaction mixture to correct the data. After 1 h reaction, the released DNA (code DNA) in the supernatant was collected and was quantified by quantitative PCR (qPCR). As shown in Figure 3b, we confirmed that the amount of released code DNA increased linearly in hypochlorite ion dose-dependent manner. In contrast, the amount of released DNA was negligible in the control experiments using Biotin-dsDNA, which did not have PS group. Thus, the system could quantify hypochlorite ion using the cleavage reaction at PS group.

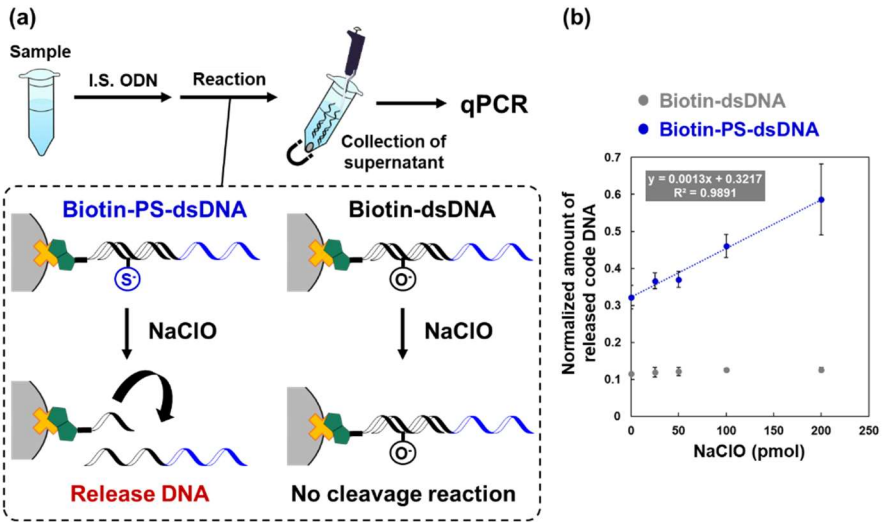

Figure 3. (a) Schematic illustration of quantification of hypochlorite ions by qPCR analysis. (b) Quantification of code DNA by qPCR analysis, 0, 25, 50, 100 or 200 pmol of NaClO was added to 5 pmol of biotin-PS-dsDNA or biotin-dsDNA. Data = mean ± SD (n = 3)

We also evaluated the effect of the position of the PS modification on the strand cleavage by hypochlorite ion. In the experiments shown above, the PS group was modified at the center of the 20 mer Biotin-PS-DNA. This was because we expected that the melting temperature (T_m) of the duplex would be low enough to release the code DNA as a result of the cleavage at the PS group. We newly designed biotin-PS(1)-DNA which has PS modification at the 5' end of the DNA. In other words, we evaluated the difference in the release rate of the code DNA after reaction depending on the position of PS modification. After preparation of two types of magnetic beads, which were modified by Biotin-PS-dsDNA or Biotin-PS(1)-dsDNA, we added 0-200 pmol sodium hypochlorite. As shown in Figure 4, we observed a linear increase of the rate of code DNA release in both cases. Moreover, no significant difference in the rate was observed between these two samples. These results indicate that the reactivity of hypochlorite ion with PS group did not change either at the center of the duplex or even at the end closest to the magnetic beads.

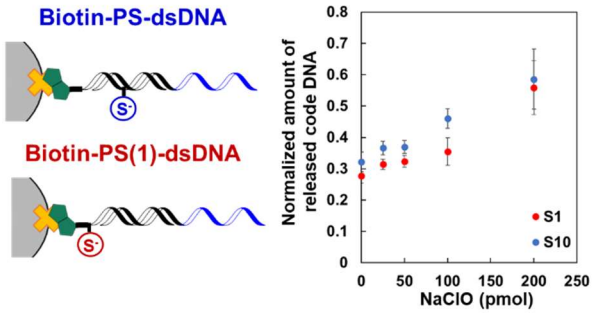

Figure 4. Quantification of code DNA by qPCR analysis, 0, 25, 50, 100, or 200 pmol of NaClO was added to 5 pmol of biotin-PS(1)-dsDNA or biotin-PS-dsDNA. Data = mean ± SD (n = 3)

In separate experiments, we evaluated the reaction selectivity of Biotin-PS-dsDNA using H_2O_2, which is the most stable and abundant ROS in the cells, instead of hypochlorite ion.[10] In addition, we evaluated whether the ODNs could react with hypochlorite ion in the presence of GSH, which functions as a reducing agent in the cell.[11] We found that the reactions of ODNs with sodium hypochlorite in the presence of H_2O_2 was the same as those in the absence of H_2O_2, and that addition of GSH resulted in a negligible effect on the reaction with hypochlorite ion. Thus, the system could work even in the presence of H_2O_2, a typical ROS, and GSH, a reducing agent in the cells (Figure 5).

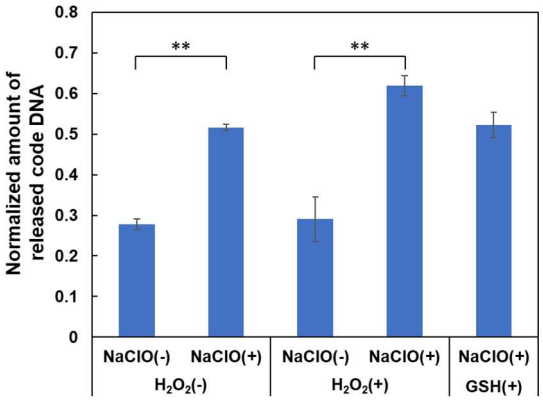

Figure 5. Quantification of code DNA by qPCR analysis, 0, or 200 pmol of NaClO was added to 5 pmol of biotin-PS-dsDNA in the presence of 0 or 200 pmol of H_2O_2 or 1 nmol GSH. Data = mean ± SD (n = 3, ** indicates $p < 0.05$)

Finally, we attempted to quantify hypochlorite ions in cell lysate. We prepared the cell lysate of mouse macrophage-like cells, RAW264.7 and added sodium hypochlorite (Figure 6a). The similar experiments using this lysate and magnetic beads labeled with Biotin-PS-dsDNA revealed that the amount of released code ODN increased as increasing amount of hypochlorite in the lysate (Figure 6b). Thus, hypochlorite in the cell lysate could be quantified in the same way as in aqueous solution.

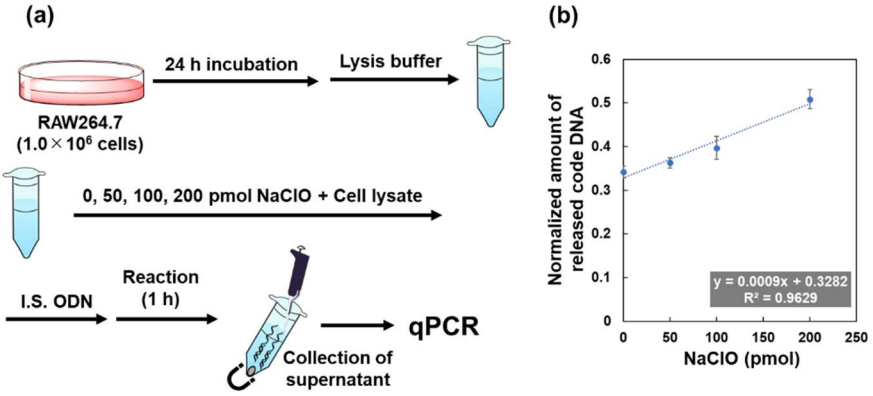

Figure 6. (a) Schematic illustration of quantification of hypochlorite with cell lysate. (b) Quantification of code DNA by qPCR analysis, 0, 50, 100, or 200 pmol of NaClO was added to 5 pmol Biotin-PS-dsDNA with cell lysate. Data = mean ± SD (n = 3)

2-3 Conclusion

In this study, we designed a molecular system by which hypochlorite ion was quantified. We prepared magnetic beads labeled by PS-modified ODNs and characterized their reaction. Addition of hypochlorite ion to the magnetic beads resulted in the DNA cleavage at the PS group. After cleavage of the DNA, the released code DNA was quantified by qPCR to quantify target hypochlorite ion. Eventually, we successfully quantified hypochlorite ion in cell lysate as well as aqueous solution, using this system. The system would be applied to integrated analysis of transcriptome, metabolome and

identification of reactive small molecules in cells. The detection and quantification of various biomolecules in cells by DNA encoding strategy is in progress.

2-4 Experimental section

General Methods

Reagents were purchased from Wako pure chemical industries, Takara Bio, Tokyo chemical industries, New England Bio Labs, and eurofins Genomics. Native polyacrylamide gel electrophoresis was carried out using an AE-8800 COSTAPOWER 3000 (ATTO). The gel image was acquired by FluoroPhoreStar 3000 (LED light irradiation 530 nm) (anatech). Quantitative PCR (qPCR) analysis and DNA hybridization were carried out with MyGo Mini S Real Time PCR (IT-IS Life Science Ltd.). DNA sequences are shown in Table 1.

Table 1. Sequences in this study

Strand name	Sequences (5' to 3')
Biotin-PS(10)-DNA	[BioON] AGGTATGTAT*GCTTAGGGTC
Biotin-PS(1)-DNA	[BioON] A*GGTATGTATGCTTAGGGTC
Biotin-DNA	[BioON] AGGTATGTATGCTTAGGGTC
Biotin-PS-DNA-FAM	[BioON] AGGTATGTAT*GCTTAGGGTC [FAM]
Biotin-DNA-FAM	[BioON] AGGTATGTATGCTTAGGGTC [FAM]
Code ODN	GAA TTC CTG TAG ATT GGA CCT TTT TTT TTT TTT TTT TTT CGA CCC TAA GCA TAC ATA CCT
Forward primer	AGG TAT GTA TGC TTA GGG TCG
Reverse primer	GAA TTC CTG TAG ATT GGA CC
Internal standard ODN	CCA GGT TAG ATG TCC TTA AGT TTT TTT TTT TTT TTT TTT TCC ATA CAT ACG AAT CCC AGC
Forward primer (for I.S. ODN)	GCT GGG ATT CGT ATG TAT GGA
Reverse primer (for I.S. ODN)	CCA GGT TAG ATG TCC TTA AG

Asterisk represents the phosphorothioate modification

Reaction monitoring of biotin-PS-dsDNA

Reaction monitoring of reaction ODN (biotin-SS-ODN) and GSH in the solution

1 μM biotin-PS-dsDNA was reacted with 0, 1, 2.5, 5.0 μM GSH in PBS for 1 h at 37°C. The reaction mixture was analyzed by 10% native-PAGE.

DNA hybridization

2 μM code ODN and biotin-PS-DNA (or PS(1)) or biotin-ODN were hybridized in milliQ water containing 5 mM $MgCl_2$. DNA was heated to 95°C for 5 min and then cooled at a rate of 0.1°C per sec to 25°C.

Binding of biotin-PS-dsDNA with streptavidin coated magnetic beads

Biotin-PS-dsDNA or biotin-dsDNA was bound with streptavidin coated magnetic beads (50 μg, Magnosphere^{TM} MS300/Streptavidin, JSR corporation) according to the manufacturer's procedure as follows. Biotin-SS-dsDNA was incubated in the binding buffer (10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 1 M NaCl, 0.05% Tween20) containing streptavidin magnetic beads for 10 min. Then, the beads were washed with binding buffer twice and suspended with the binding buffer (20 μL/sample).

Evaluation of phosphorothioate (PS) dependent reaction with hypochlorite ions on magnetic beads

Biotin-PS-dsDNA or biotin-dsDNA (5 pmol) labeled magnetic beads (50 μg) were washed with PBS (20 μL). Then, NaClO (0, 25, 50, 100 or 200 pmol) and internal standard ODN (5 pmol) in PBS (20 μL) was mixed with magnetic beads and incubated for 1 h at 37°C by gentle shaking. The supernatant (10 μL) was collected using magnet.

After dilution using milliQ water (×10,000), the sample was quantified using qPCR. qPCR was performed according to the manufacturer's procedure using Luna Universal qPCR Master Mix (NEW ENGLAND Bio Labs): 2 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C.

Evaluation of location of PS modification

Biotin-PS-dsDNA (or PS(1)) (5 pmol) labeled magnetic beads (50 μg) were washed with PBS (20 μL). Then, NaClO (0, 25, 50, 100 or 200 pmol) and internal standard ODN (5 pmol) in PBS (20 μL) was mixed with magnetic beads and incubated for 1 h at 37°C by gentle shaking. The supernatant (10 μL) was collected using magnet. After dilution using milliQ water (×10,000), the sample was quantified using qPCR. qPCR was performed according to described above.

Quantification of hypochlorite ion in the presence of other ROS and reducing agent

Biotin-PS-dsDNA (5 pmol) labeled magnetic beads (50 μg) were washed with PBS (20 μL). Then, NaClO (0 or 200 pmol) and internal standard ODN (5 pmol) in PBS (18 μL) was mixed with magnetic beads and added 100 μM H_2O_2 (0 or 200 pmol, 2 μL) or 500 μM GSH (1nmol, 2 μL) incubated for 1 h at 37°C by gentle shaking. The supernatant (10 μL) was collected using magnet. After dilution using milliQ water (×10,000), the sample was quantified using qPCR. qPCR was performed according to described above.

Cell culture

Mouse macrophage-like cells, RAW264.7 was cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin. The cells were

maintained at 37°C in 5% CO_2 / 95% air and were kept in a logarithmic growth phase by routine passages every 2−3 days. Prior to the use of cells, the densities of cells were determined using a hemocytometer.

Quantification of hypochlorite ions in cell lysate

RAW264.7 cells (1.0×10^6 cells per well) were seeded onto 6 well plate in 3 mL DMEM. Plate was maintained at 37°C in 5% CO_2 / 95% air incubator for 24 h. After washing with D-PBS (-) twice, lysis buffer (10 mM Tris-HCl (pH7.4), 2 mM EDTA, 0.05% Triton-X) was added into each well and incubated for 15 min on ice. Then, cell lysates (500 μL) were centrifuged at 12,000 g for 3 min at 4°C, and collected supernatant.

Biotin-PS-dsDNA (5 pmol) labeled magnetic beads (50 μg) were washed with PBS (20 μL). Then, NaClO (0, 50, 100 or 200 pmol) and internal standard ODN (5 pmol) in PBS (16 μL) was mixed with cell lysate (4 μL) magnetic beads and incubated for 1 h at 37°C by gentle shaking. The supernatant (10 μL) was collected using magnet. After dilution using milliQ water (×10,000), the sample was quantified using qPCR. qPCR was performed according to described above.

2-5 Reference

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Chapter 3

DNA encoding strategy for the identification and quantification of poorly reactive target metabolite using phenylboronic acid-tethered oligodeoxynucleotides

Abstract

Information about poorly reactive metabolites, lactate and glucose could be encoded into DNA sequences. The sequential reaction steps, including H_2O_2 generation between the target metabolites and their corresponding oxidase, the reaction between phenylboronic acid-tethered oligonucleotide and H_2O_2, and biotinylation of product DNA allow the DNA encoding strategy to identify and quantify the target metabolites.

3-1 Introduction

DNA encoding technology is experiencing rapid advancements, particularly in ligand screening[1] and biological target detection.[2] One significant application owing to the development of DNA encoding strategies for cellular information is the utilization of next-generation sequencers (NGS) for single-cell transcriptome analysis. This approach allows for the analysis of a large number of cells.[3] Moreover, researchers are exploring DNA encoding strategies for various biomolecules beyond mRNA. For instance, using a complex of an antibody and DNA makes it possible to broaden the range of targets, such as cell surface antigens, by reading the sequences bound to them.[4] These DNA coding methods offer the advantage of simultaneous transcriptome analysis, making them highly valuable in research.

In the medical field, metabolites are actively studied for their role as disease biomarkers.[5] Methods that can convert metabolite information into DNA sequences are highly valuable as they enable simultaneous analysis with transcriptome analysis. Recently, we introduced a methodology for encoding metabolite information into DNA sequences.[6] Specifically, we focused on the metabolite glutathione (GSH) and used the high reactivity of its thiol group to achieve DNA encoding. This encoding was achieved by designing the DNA loaded on magnetic beads to be released during a disulfide bond exchange reaction. While we successfully encoded GSH information into DNA sequence, it is important to note that this DNA encoding strategy is limited to highly reactive metabolites with reactive substituents, such as GSH. The majority of metabolites lack such substituents; therefore, this strategy cannot be applied to them.

Given the research context and the need to broaden our DNA encoding strategy, we aimed to extend the detection capability to other types of target metabolites.

Specifically, we focused on poorly reactive metabolites, such as lactate and glucose.[7] These metabolites are of significant interest due to their central roles in the energy metabolism of cancer cells.[8] However, encoding these metabolites into DNA has been challenging thus far, primarily because they lack reactive substituents, such as thiol and amino groups, which can readily react with the molecular system.

To encode and detect these metabolites, we designed a molecular system utilizing the oxidase reaction,[9] which produces a highly reactive molecule, hydrogen peroxide (H_2O_2), during the reaction between metabolites (lactate and glucose) and their corresponding oxidases (lactate oxidase (LOx) and glucose oxidase (GOx)) (Figure 1a). We prepared **PB-dsDNA**, formed from three DNA strands (*PB-DNA*, *code DNA*, and *SS-DNA*), and immobilized it on the beads. The *PB-DNA* was terminally modified with phenylboronic acid pinacol ester (PB), which highly selectively reacted with H_2O_2 in the solution (Figure 1b). H_2O_2-mediated oxidation converted the phenylboronic ester into phenol moiety[10] and released CO_2 and quinone methide to give an amino group.[11] The code DNA was the strand to encode target metabolites and sample information. The *SS-DNA* was responsible for the linkage of DNA units with beads and the release of them from the beads via disulfide bond cleavage.

We followed five steps for encoding metabolites into DNA and quantifying them (Figure 1a). The initial step was the reaction of phenylboronic acid pinacol ester unit in **PB-dsDNA** with H_2O_2 to form **NH_2-dsDNA**. **PB-dsDNA** was converted to **NH_2-dsDNA** by H_2O_2 via decarboxylation (Figure 1b).[10, 11] The second step was biotin labeling of **NH_2-dsDNA** to obtain **Biotin-dsDNA**. The third step was DTT treatment to release **dsDNAs** (**PB-dsDNA** and **Biotin-dsDNA**) from the beads via disulfide bond cleavage at *SS-DNA*. The fourth step was selective collection of **Biotin-dsDNA** by streptavidin-coated

magnetic beads (SA beads). The fifth step was heating to collect the *code DNA* from the beads, which represent the amount of target metabolite (Figure 1c).

The *PB-DNA* was synthesized by coupling a phenylboronic acid derivative with amine-terminated DNA (*NH_2-DNA*). The *PB-DNA* derivative with Cy5 fluorophore (*PB-DNA-Cy5*) was also synthesized separately to follow the enzymatic reaction by using HPLC.

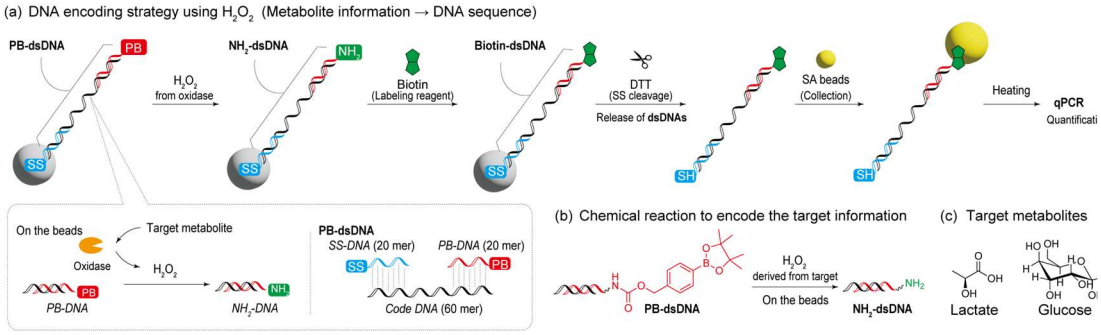

Figure 1 (a) Schematic illustration to encode target metabolite information into DNA sequences using its oxidases and phenylboronic acid-tethered oligonucleotides. (b) Chemical reaction to encode the target metabolite information using the H_2O_2 derived from the target metabolite. (c) Structure of target metabolites in this study (lactate and glucose).

3-2 Result & Discussion

First, we evaluated the reaction between *PB-DNA-Cy5* and H_2O_2 generated by oxidases (Figure 2a). Among the two target metabolites, we selected lactate as a model compound and monitored the reaction in the samples containing LOx and different concentrations of lactate using HPLC analysis. The results, as shown in Figure 2b, confirmed the formation of *NH_2-DNA-Cy5* from *PB-DNA-Cy5*, as evidenced by comparing it with authentic *NH_2-DNA-Cy5* in HPLC analyses (Figure 3). Although the prepared *PB-DNA-Cy5* contains *NH_2-DNA-Cy5* as an impurity, the yield of *NH_2-DNA-Cy5* exhibited a dose-dependent increase with lactate concentration (Figure 2c). In

addition, the *PB-DNA-Cy5* was stable in the phosphate buffer for 1 h (Figure 4). To elucidate the significance of H_2O_2 in the conversion process from *PB-DNA-Cy5* to *NH_2-DNA-Cy5*, we introduced catalase into the reaction solution. The addition of catalase dramatically suppressed the conversion, indicating the pivotal role of H_2O_2 production in the transformation (Figure 5).

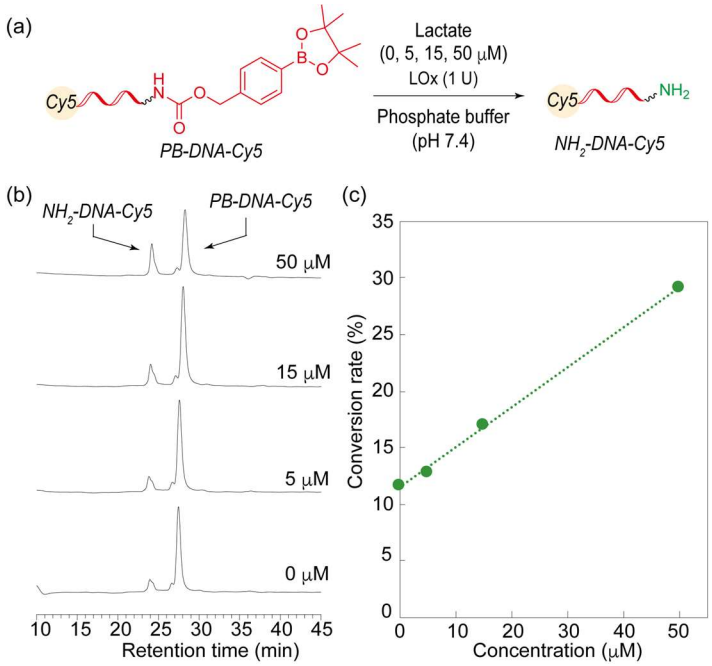

Figure 2 (a) Chemical reaction between *PB-DNA-Cy5* and H_2O_2 derived from lactate. (b) Reaction monitoring using HPLC. Briefly, 3 μM *PB-DNA-Cy5* was reacted with 0, 5, 15, and 50 μM lactate and 1 U LOx in 250 mM phosphate buffer (pH 7.4) for 1 h. (c) A conversion rate (*NH_2-DNA-Cy5*/ (*PB-DNA-Cy5* + *NH_2-DNA-Cy5*)) was determined using each peak area derived from Cy5 absorbance.

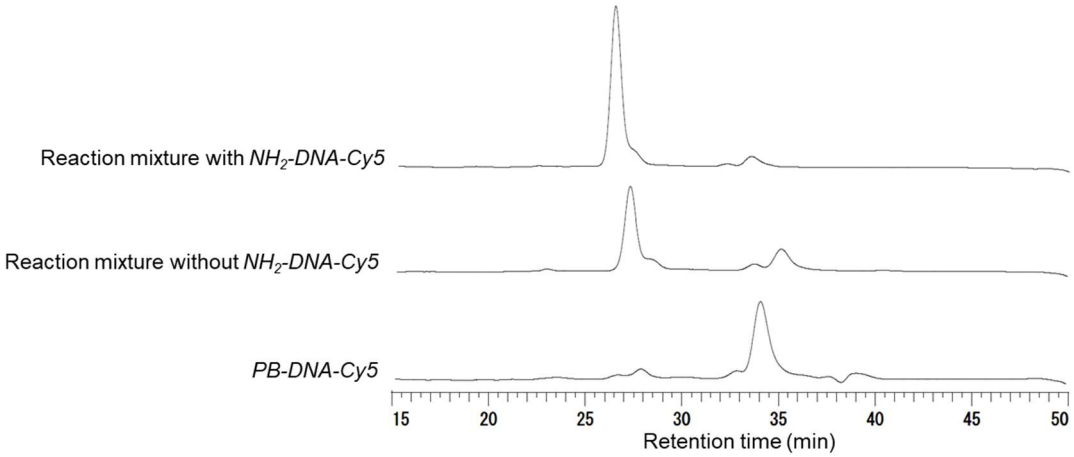

Figure 3. HPLC chart of 5 μM *PB-DNA-Cy5* incubated with 1 mM lactate and 1 U LOx in 250 mM PB (pH7.4, 10 μL) for 1 h at 37°C with or without 50 pmol *NH_2-DNA-Cy5*.

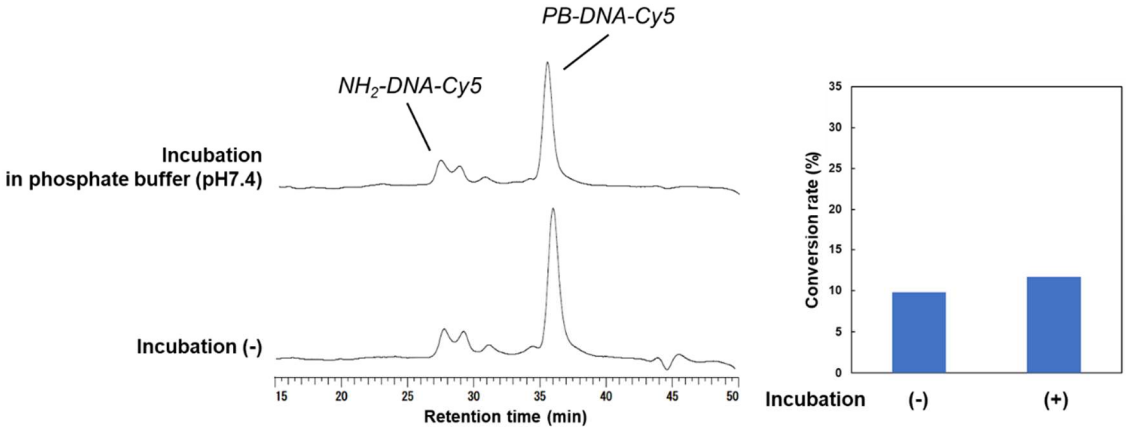

Figure 4. HPLC chart of 3 μM *PB-DNA-Cy5* incubated in 250 mM PB (pH7.4) for 0 or 1 h at 37°C.

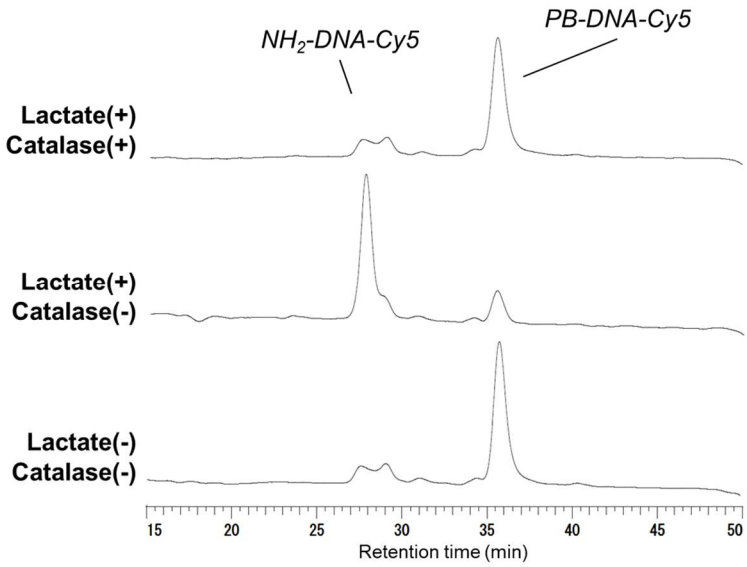

Figure 5. HPLC chart of 3 μM *PB-DNA-Cy5* incubated with 1 mM lactate, 1 U LOx and 0 or 10 U catalase in 250 mM PB (pH7.4) for 1 h at 37°C.

We proceeded with the evaluation of several steps: biotin-labeling of **NH_2-dsDNA**, release of the DNA unit from the beads through DTT treatment, and quantification of the released DNA via qPCR (Figure 6a, 7a). In this evaluation, we loaded varying amounts of **NH_2-dsDNA** (test sample) onto the beads. Next, we introduced a biotinylating reagent (Biotin-AC_5 Sulfo-OSu, labeling reagent) and allowed the sample to incubate for 1 h. Following the reaction, we replaced the supernatant with a reducing solution containing DTT, which facilitated the cleavage of the disulfide bond and released the **dsDNAs** from the beads. The resulting **Biotin-dsDNA** was selectively collected using streptavidin-coated magnetic beads (SA beads). Subsequently, the supernatant underwent heating, and the *code DNA* present was quantified using qPCR. To ensure accurate quantification, we introduced another **NH_2-dsDNA** (internal standard), DNA strand with different sequence (*IS DNA*) onto the beads. *IS DNA* were used to correct for the efficiency of **Biotin-dsDNA** collection and *code DNA* release. Using the

ratio of *code DNA* amount to *IS DNA* amount, we estimated the amount of **NH₂-dsDNA** (test sample) (Figure 6b, 7b, c). The results of quantification for **NH₂-dsDNA** on the beads demonstrated accurate quantification up to 500 fmol, with an approximate collection rate of 4% for **NH₂-dsDNA**. Furthermore, we observed that the efficiency of biotin labeling reached its upper limit when approximately 10 nmol of the biotinylating agent was added to **NH₂-dsDNA** on the beads (Figure 8).

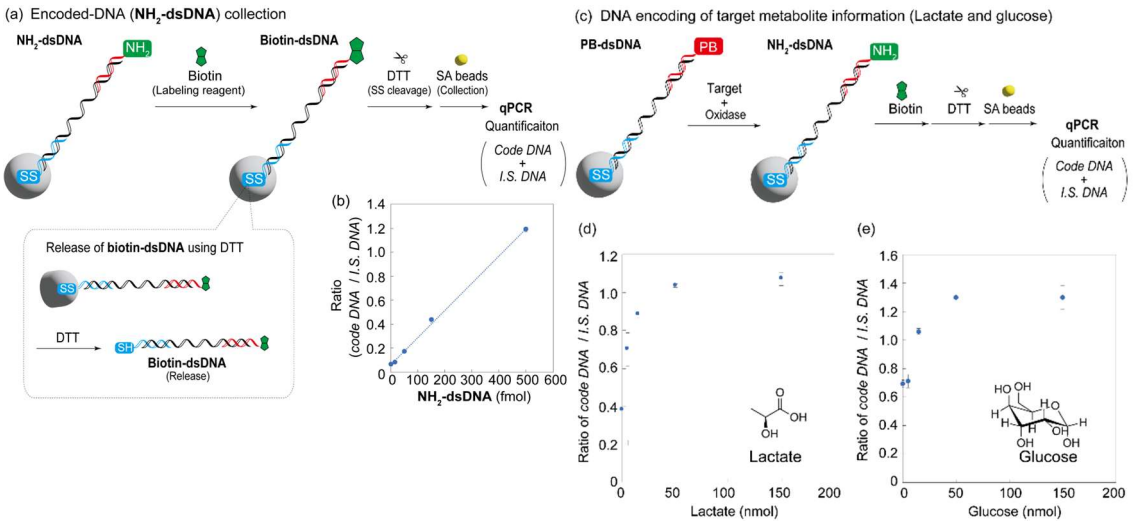

Figure 6 (a) Schematic illustration to collect the encoded DNA (**NH₂-dsDNA**) and disulfide cleavage reaction using the reducing solution containing DTT. (b) Amount of *code DNA* after collection (Biotin labeling→DTT treatment→collection using streptavidin-coated magnetic beads). Confirmation of collection ability by quantifying *code DNA*. (c) Schematic illustration to encode the target molecule information. The ratio of *code DNA*/*IS DNA* after DNA encoding for (d) lactate and (e) glucose. Data = mean ± SD (n = 3).

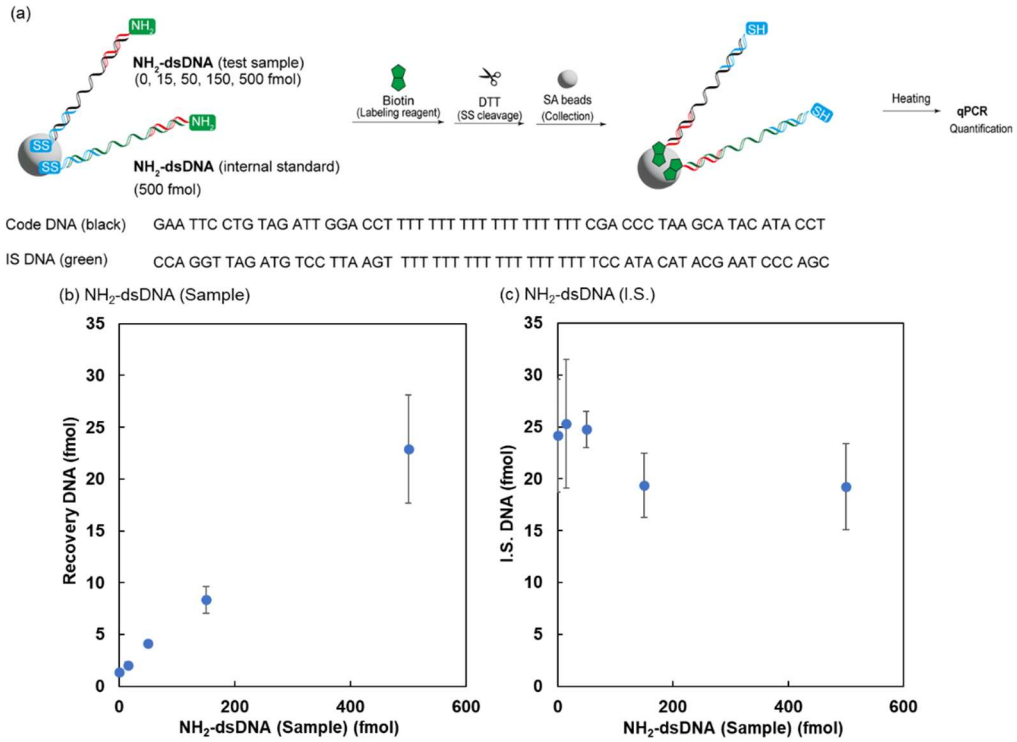

Figure 7. Collection and quantification of **NH_2-dsDNA (test sample and I.S.)** using the biotin labelling. (a) Schematic illustration to collect 0, 15, 50, 150 or 500 fmol of **NH_2-dsDNA (test sample)** and 500 fmol of **NH_2-dsDNA (I.S.)** on magnetic beads. Quantification of collected (b) **NH_2-dsDNA (test sample)** and (c) **NH_2-dsDNA (I.S.)**. Data = mean ± SD (n = 3).

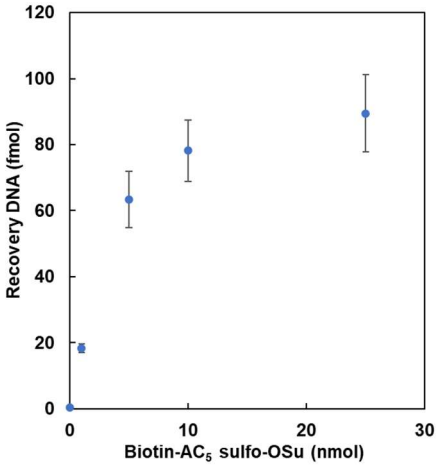

Figure 8. Evaluation of biotinylation of **NH_2-dsDNA** on the magnetic beads. Quantification of 1 pmol of **NH_2-dsDNA** on magnetic beads using 0, 2.5, 5.0, 10, 25 nmol Biotin-AC_5 sulfo-OSu. Data = mean ± SD (n = 3).

With a suitable molecular system to collect and quantify **NH_2-dsDNA**, we attempted to apply the present DNA encoding system to the detection of the target metabolites (Figure 6c). After constant amounts of **PB-dsDNA** and **NH_2-dsDNA** as internal standard were loaded on the beads, a sample solution containing the target metabolites (lactate and glucose), and their corresponding oxidases (LOx and GOx) were mixed. Similar to the above experiments, **NH_2-dsDNA** was labeled with biotin and collected using the SA beads. After heating, the code DNA was quantified using qPCR. As shown in Figure 6d and 6e, as the concentration of target metabolites increased, the ratio of *code DNA* and *I.S. DNA*, which represents concentration of **NH_2-dsDNA** (**target encoded dsDNA**), increased in a dose-dependent manner. Thus, the amount of target metabolites in the sample could be quantified using this system and suitable oxidases.

To gain further insights into the metabolic processes occurring in cancer cells, we conducted analyses of glucose consumption and lactate production. Cancer cells exhibit enhanced glycolytic pathway activity, known as the Warburg effect, to generate energy.[8] To demonstrate the versatility of our molecular system, we monitored the levels of lactate and glucose in the cell culture medium, which fluctuate with cell growth (Figure 9a).[12] HeLa cells were seeded, and after 48 h of incubation the medium was analyzed using our system (Figure 9b, c). Notably, in the presence of cells, the level of **NH_2-dsDNA** (**lactate information encoded dsDNA**) increased in the medium, while the level of **NH_2-dsDNA** (**glucose information encoded dsDNA**) decreased. To validate these findings, we performed a separate experiment using a commercially available kit to quantitatively measure glucose and lactate concentrations in the medium, confirming a similar trend in the changes of the target metabolite concentrations (Figure 10). Although the Z prime

value was low (Z' = 0.2),[13] our system may be improved by suppressing the background signal under the condition without the lactate.

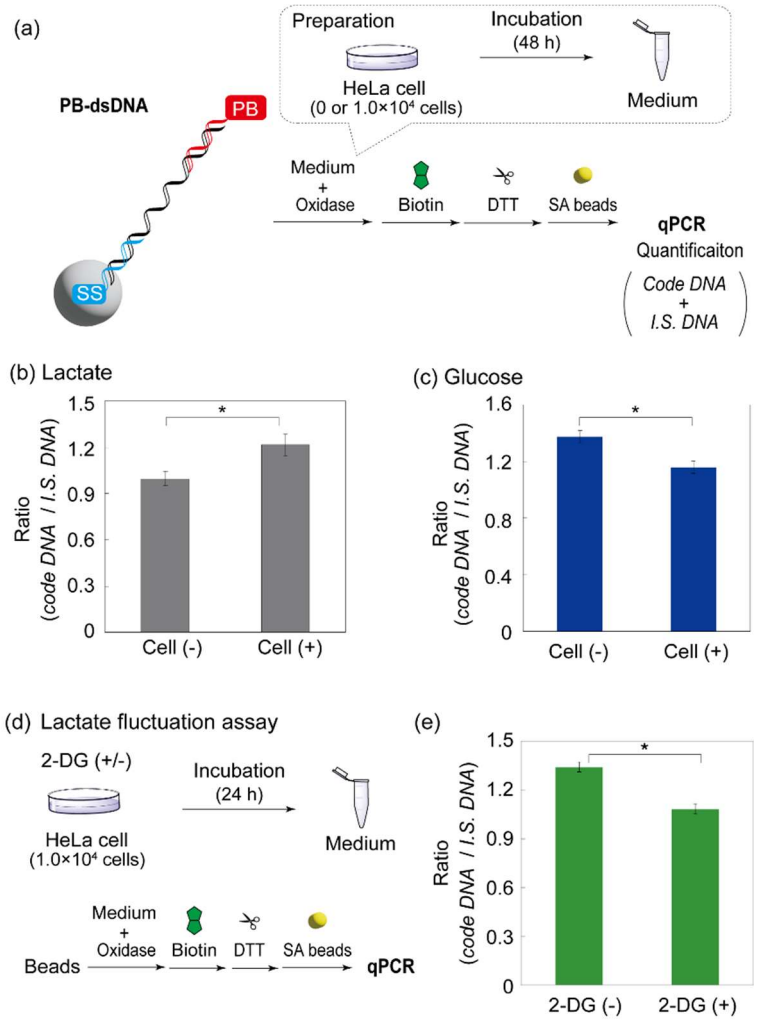

Figure 9 (a) Schematic illustration to detect the target metabolite in the cultured medium. The ratio of *code DNA*/*IS DNA* after DNA encoding (b) lactate and (c) glucose. (d) Schematic illustration of lactate fluctuation assay and beads assay to monitor lactate levels. (e) The ratio of *code DNA*/*IS DNA* after DNA encoding of lactate in the cell culture medium under glycolysis inhibition condition. Data = mean ± SD (n = 3). *p* values were obtained by using a Student's t-test: $*p < 0.05$.

Furthermore, we utilized our system to track the change in lactate production by inhibiting glycolysis with 2-deoxy-D-glucose (2-DG).[14] After treating HeLa cells with 2-DG, a significant decrease in lactate levels in the medium was observed (Figure 9d, e, 11). These results strongly indicate that the metabolic processes of cells can be effectively monitored using our DNA encoding system.

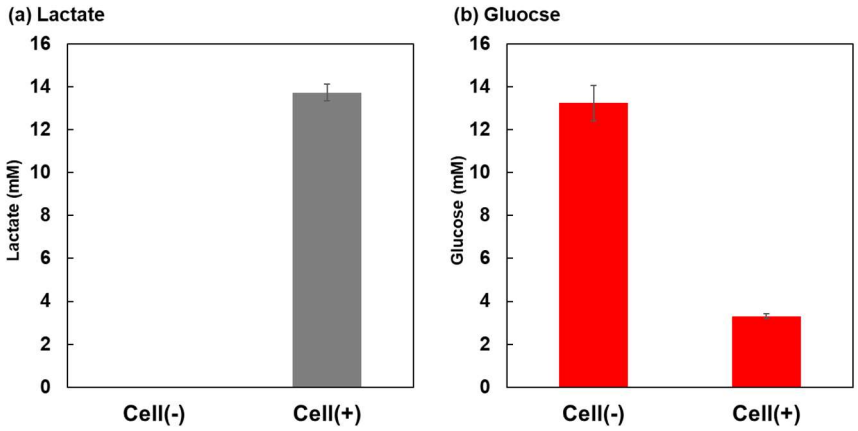

Figure 10. Quantification of lactate and glucose in the culture medium by commercially available assay kit. Quantification of (a)lactate and (b)glucose in cell culture medium of HeLa cells (0 or 1×10^4 cells/well) incubated for 48 h by commercially available assay kit. Data = mean ± SD (n = 3).

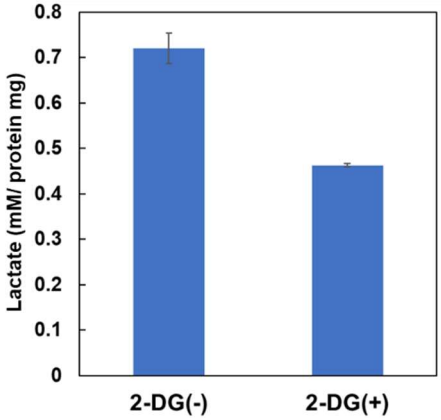

Figure 11. Quantification of lactate in the culture medium under glycolysis inhibition condition using commercially available assay kit. Quantification of lactate in cell culture medium of HeLa cells (1.0×10^4 cells/well) incubated with 0 or 5 mM 2-Deoxy-D-glucose for 24 h by commercially available assay kit. Data = mean ± SD (n = 3).

3-3 **Conclusion**

In conclusion, we demonstrated the successful identification and quantification of poorly reactive metabolites, specifically lactate and glucose, using a DNA encoding strategy. This approach involves the preparation of beads carrying phenylboronic acid-tethered oligonucleotides, which undergo reactions with H_2O_2 generation from the reaction between the target metabolite and its corresponding oxidase, enabling their identification. Through a series of reaction steps on the beads, including H_2O_2 generation, conversion into **NH_2-dsDNA**, biotinylation, reductive cleavage of **dsDNAs** from the beads, and quantification of the released **Biotin-dsDNA** using qPCR, we could detect and quantify the target metabolites. We successfully detected glucose and lactate and further demonstrated the ability to monitor changes in lactate concentration in cell culture medium. Therefore, our DNA encoding system provides a valuable tool for the identification and quantification of target metabolites.

3-4 Experimental section

General Methods

Reagents were purchased from Wako pure chemical industries, Sigma Aldrich, Nacalai Tesque, Takara Bio, Dojindo, Tokyo chemical industries, New England BioLabs, and eurofins Genomics. Analytical and preparative high-performance liquid chromatographies were performed on a D2000 HPLC system and L7000 HPLC system (HITACHI) with reversed phase column (InerrtsilvODS-3, GL Science Inc., ϕ 1.0 mm×250 mm (particle size; 5 μm), ϕ 4.6 mm×250 mm (particle size; 5 μm)). MALDI-TOF mass spectrometry were recorded on an AXIMER-LNR spectrometer

(SHIMADZU). DNA hybridization and quantitative PCR (qPCR) analysis was carried out with MyGo Mini S Real Time PCR (IT-IS Life Science Ltd.). Lactate assay (Lactate Assay Kit-WST, L256, DOJINDO), glucose assay (Glucose Assay Kit-WST, G264, DOJINDO) and protein assay (Wako) were recorded on a xMarkTM microplate absorbance spectrophotometer (BIO RAD). DNA sequences are shown in Table 1.

Table 1. DNA sequences in this study

Strand name	Sequences (5' to 3')
NH_2-DNA	GGT CCA ATC TAC AGG AAT TC-NH_2
NH_2-DNA-Cy5	Cy5-GGT CCA ATC TAC AGG AAT TC-NH_2
NH_2-DNA (for SS-DNA)	NH_2-AGG TAT GTA TGC TTA GGG TC
Code DNA	GAA TTC CTG TAG ATT GGA CCT TTT TTT TTT TTT TTT TTT CGA CCC TAA GCA TAC ATA CCT
Internal standard DNA	CCA GGT TAG ATG TCC TTA AGT TTT TTT TTT TTT TTT TTT TCC ATA CAT ACG AAT CCC AGC
NH_2-I.S. DNA	CTT AAG GAC ATC TAA CCT GG-NH_2
NH_2-I.S. DNA (for SS-DNA)	NH_2-GCT GGG ATT CGT ATG TAT GG
Forward primer	AGG TAT GTA TGC TTA GGG TCG
Reverse primer	GAA TTC CTG TAG ATT GGA CC
Forward primer (for I.S. DNA)	GCT GGG ATT CGT ATG TAT GGA
Reverse primer (for I.S. DNA)	CCA GGT TAG ATG TCC TTA AG

Synthesis

Synthesis of *PB-DNA*

The solution of phenylboronic acid N-hydroxysuccinimide ester (500 mM in DMSO, 10 μmol) were added the solution of *NH_2-DNA* (1 mM in milliQ, 5 nmol) in 100 mM pH8.7 carbonate-bicarbonate buffer containing 50% DMSO and incubated at room temperature for 2 h. The reaction mixture was purified using Micro Bio-Spin™ 6 Columns (Bio-Rad) and milliQ water as an elution solution. The concentration of the oligomer was determined by complete digestion with alkaline phosphatase (AP), nuclease P1 (PI) and phosphodiesterase I at 37°C for overnight. Identities of synthesized oligomers were confirmed by MALDI-TOF MS spectrometry using saturated 3-hydroxypicolinic acid in 100 mM diammonium hydrogen citrate solution as a matrix ([M-H]$^-$ calcd. 6566.26, found. 6566.03). The purity of synthesized oligomer was confirmed by monitoring of UV

absorbance at 260 nm at a flow rate of 0.6 (analysis) $mLmin^{-1}$ with a 0–25% (over 50 min) gradient of acetonitrile (ACN) / triethylammonium acetate buffer (TEAA buffer) (100 mM, pH 7.0) (Figure S1a).

Synthesis of *PB-DNA-Cy5*

PB-DNA-Cy5 was synthesized as described above. Identities of synthesized oligomers were confirmed by MALDI-TOF MS spectrometry using saturated 3-hydroxypicolinic acid in 100 mM diammonium hydrogen citrate solution as a matrix ($[M-H]^-$ calcd. 7098.51, found. 7099.47). The purity of synthesized oligomer was confirmed by monitoring of UV absorbance at 640 nm at a flow rate of 0.6 (analysis) $mLmin^{-1}$ with a 20–24% (0–10 min) and 24–26% (10–40 min), 26–100% (40–50 min) gradient of ACN/TEAA buffer (100 mM, pH 7.0) (Figure S1b).

Synthesis of *SS-DNA*

The solution of Biotin-SS-sulfo-OSu (16.5 mM in milliQ, 2.1 μmol) were added the solution of *NH_2-DNA* (1 mM in milliQ, 5.0 nmol) in 100 mM pH8.7 carbonate-bicarbonate buffer and incubated at room temperature for 20 h. The reaction mixture was purified using Micro Bio-Spin™ 6 Columns (Bio-Rad) and milliQ water as an elution solution. Moreover, eluted sample was purified by HPLC again. The column eluents were monitored by the UV absorbance at 260 nm at a flow rate of 3.0 (preparative) $mLmin^{-1}$ with a 10–35% (over 30 min, for synthesis, *SS-DNA*) gradient of ACN/TEAA buffer (100 mM, pH 7.0). The concentration of the oligomer was determined by complete digestion with AP, PI and phosphodiesterase I at 37°C for overnight. Identities of synthesized oligomers were confirmed by MALDI-TOF MS spectrometry. ((For sample) $[M-H]^-$

calcd. 6767.21, found. 6767.66, (For internal standard) $[M-H]^-$ calcd. 6783.21, found. 6782.78). The purity of synthesized oligomer was confirmed by monitoring of UV absorbance at 260 nm at a flow rate of 0.6 (analysis) $mLmin^{-1}$ with a 10–60% (over 50 min) gradient of ACN/TEAA buffer (100 mM, pH 7.0) (Figure S5).

Reaction monitoring of *PB-DNA-Cy5*

Reaction monitoring of *PB-DNA-Cy5* with lactate and LOx in the solution

3 μM *PB-DNA-Cy5* was reacted with 0, 5, 15, 50 μM lactate and 1 U LOx in PB (250 mM, pH 7.4) for 1 h at 37°C.

Confirmation of *NH_2-DNA* production using authentic sample

5 μM *PB-DNA-Cy5* was reacted with 1 mM lactate and 1 U LOx in 250 mM PB (pH 7.4) for 1 h at 37°C. The reaction mixture (10 μL) was analyzed by HPLC with or without 50 pmol *NH_2-DNA-Cy5*.

Stability of PB-DNA-Cy5 in the phosphate buffer

3 μM *PB-DNA-Cy5* was incubated in 250 mM PB (pH 7.4) for 0 or 1 h at 37°C. The reaction mixture (20 μL) was analyzed by HPLC.

Inhibition of H_2O_2 reaction using the catalase

3 μM *PB-DNA-Cy5* was reacted with 1 mM lactate, 1 U LOx and 0 or 10 U catalase in 250 mM PB (pH 7.4) for 1 h at 37°C.

The reaction mixtures were analyzed by HPLC. The column eluents were monitored by the UV absorbance at 640 nm at a flow rate of 0.6 (analysis) $mLmin^{-1}$ with a 20–24% (0–10 min) and 24–26% (10–40 min), 26–100% (40–50 min) gradient of ACN/TEAA buffer (100 mM, pH 7.0).

Reaction on magnetic beads

DNA hybridization

1 μM *code DNA*, *SS-DNA* and *PB-DNA* (for **PB-dsDNA**) or *NH_2-DNA* (for **NH_2-dsDNA**) were hybridized in PBS containing 5 mM $MgCl_2$. DNA was heated to 95°C for 5 min and then cooled at a rate of 1°C per min to 25°C.

Binding of PB-dsDNA or NH_2-dsDNA with streptavidin coated magnetic beads

PB-dsDNA or **NH_2-dsDNA** was bound with streptavidin coated magnetic beads (50 μg/sample, Magnosphere^{TM} MS300/Streptavidin, JSR corporation) according to the manufacturer's procedure as follows. Each **dsDNA** (**PB-dsDNA** or **NH_2-dsDNA**) was incubated in the binding buffer (10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 1 M NaCl, 0.05% Tween20) containing streptavidin magnetic beads for 10 min. Then, the beads were washed with binding buffer twice and suspended with the binding buffer (20 μL/sample).

Collection and quantification of NH_2-dsDNA using biotin labelling

After removal of supernatant, **NH_2-dsDNA** (0, 15, 50, 150, 500 fmol, for sample), **dsDNA** (500, 485, 450, 350, 0 fmol, for sample, without *NH_2-DNA*) and **NH_2-dsDNA** (500 fmol, for I.S.) labeled magnetic beads (50 μg) were washed with 200 mM carbonate-bicarbonate buffer (pH 8.7, 50 μL). Then, biotin-AC_5 sulfo-OSu (10 nmol, Dojindo) in

200 mM carbonate-bicarbonate buffer (pH 8.7, 20 μL) was reacted with **NH_2-dsDNA** labeled magnetic beads and incubated for 1 h at room temperature by gentle shaking. The magnetic beads were washed with the binding buffer (50 μL, twice) and 250 mM PB (pH 8.4, 50 μL). After removal of supernatant, 5 mM DTT in 250 mM PB (pH 8.4, 20 μL) was reacted with the magnetic beads for 1 h at room temperature by gentle shaking to release the **dsDNAs**. **Biotin-dsDNA** in the supernatant (20 μL) was bound with fresh magnetic beads as described above. After removal of supernatant, **Biotin-dsDNA** labeled magnetic beads were washed with milliQ (100 μL) and heated at 95°C for 5 min in the fresh milliQ (50 μL). After cooling on ice (15 sec), the collected supernatant was diluted with milliQ water (×100), the sample was quantified using qPCR.

Evaluation of biotinylation of NH_2-dsDNA on magnetic beads

After removal of supernatant, **NH_2-dsDNA** (1 pmol) labeled magnetic beads (50 μg) were washed with 200 mM carbonate-bicarbonate buffer (pH 8.7, 50 μL). Then, biotin-AC_5 sulfo-OSu (0, 1, 5, 10 or 25 nmol, Dojindo) in 200 mM carbonate-bicarbonate buffer (pH 8.7, 20 μL) was reacted with **NH_2-dsDNA** labeled magnetic beads and incubated for 1 h at room temperature by gentle shaking. The magnetic beads were washed with the binding buffer (50 μL, twice) and 250 mM PB (pH8.4, 50 μL). After removal of supernatant, 5 mM DTT in 250 mM PB (pH8.4, 20 μL) was reacted with the magnetic beads for 1 h at room temperature by gentle shaking to release the **dsDNAs**. **Biotin-dsDNA** in the supernatant (20 μL) was bound with fresh magnetic beads as described above. After removal of supernatant, **Biotin-dsDNA** labeled magnetic beads were washed with milliQ (100 μL) and heated at 95 °C for 5 min in the fresh milliQ (50 μL). After cooling on ice

(15 sec), the collected supernatant was diluted with milliQ water (×100), the sample was quantified using qPCR.

Quantification of lactate and glucose with PB-dsDNA labeled magnetic beads

After removal of supernatant, **PB-dsDNA** (500 fmol) and **NH_2-dsDNA** (500 fmol, I.S.) labeled magnetic beads (50 μg) were washed with 250 mM PB (pH7.4, 50 μL). Then, lactate (0, 5, 15, 50, 150 nmol) and lactate oxidase (LOx, 1 U) or glucose (0, 5, 15, 50, 150 nmol) and glucose oxidase (GOx, 20 U) in 250 mM PB (pH 7.4, 20 μL) was reacted with **PB-dsDNA** labeled magnetic beads and incubated for 1 h at 37°C. After removal of supernatant, beads were washed with 200 mM carbonate-bicarbonate buffer (pH 8.7, 50 μL). Then, biotin-AC_5 sulfo-OSu (10 nmol, Dojindo) in 200 mM carbonate-bicarbonate buffer (pH 8.7, 20 μL) was reacted with **NH_2-dsDNA** labeled magnetic beads and incubated for 1 h at room temperature by gentle shaking. The magnetic beads were washed with the binding buffer (50 μL, twice) and 250 mM PB (pH8.4, 50 μL). After removal of supernatant, 5 mM DTT in 250 mM PB (pH8.4, 20 μL) was reacted with the magnetic beads for 1 h at room temperature by gentle shaking to release the **dsDNAs**. **Biotin-dsDNA** in the supernatant (20 μL) was bound with fresh magnetic beads as described above. After removal of supernatant, **Biotin-dsDNA** labeled magnetic beads were washed with milliQ (100 μL) and heated at 95°C for 5 min in the fresh milliQ (50 μL). After cooling on ice (15 sec), the collected supernatant was diluted with milliQ water (×100), the sample was quantified using qPCR.

qPCR quantification

qPCR was performed according to the manufacturer's procedure using Luna Universal qPCR Master Mix (NEW ENGLAND Bio Labs): 2 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C.

Quantification of lactate and glucose in the cell culture medium

Cell culture

Human cervix epithelial adenocarcinoma HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS). The cells were maintained at 37°C in 5% CO_2 / 95% air and were kept in a logarithmic growth phase by routine passages every 2−3 days. Prior to the use of cells, the densities of cells were determined using a hemocytometer.

Quantification of lactate and glucose in cell culture medium with PB-dsDNA labeled magnetic beads

HeLa cells (0 or 1.0×10^4 cells/well) were seeded onto 96 well plate in DMEM (10% FBS, 1% PS). After 24 h incubation, cells were washed with D-PBS and replaced Opti-MEM (100 μL). After 48 h incubation, cell culture medium was collected to quantify the lactate and glucose using beads assay and colorimetric assay.

Quantification of lactate in the culture medium under glycolysis inhibition condition

HeLa cells (1.0×10^4 cells/well) were seeded onto 96 well plate in D-MEM (10% FBS, 1% PS). After 24 h incubation, cells were washed with D-PBS and replaced 0 or 5 mM

2-deoxy-D-glucose (2-DG) in Opti-MEM (100 μL). After 24 h incubation, culture medium was collected to quantify the lactate using beads assay and colorimetric assay. After removal of supernatant, **PB-dsDNA** (500 fmol) and **NH_2-dsDNA** (500 fmol, I.S.) labeled magnetic beads (50 μg) were washed with 250 mM PB (pH7.4, 50 μL). Then, 286 mM PB (pH7.4, 17.5 μL) containing LOx (1 U) or GOx (20 U) and cell culture medium (2.5 μL) was mixed with **PB-dsDNA** labeled magnetic beads and incubated for 1 h at 37 °C. After removal of supernatant, beads were washed with 200 mM carbonate-bicarbonate buffer (pH 8.7, 50 μL). Then, biotin-AC_5 sulfo-OSu (10 nmol, Dojindo) in 200 mM carbonate-bicarbonate buffer (pH 8.7, 20 μL) was reacted with **NH_2-dsDNA** labeled magnetic beads and incubated for 1 h at room temperature by gentle shaking. The magnetic beads were washed with the binding buffer (50 μL, twice) and 250 mM PB (pH8.4, 50 μL). After removal of supernatant, 5 mM DTT in 250 mM PB (pH8.4, 20 μL) was reacted with the magnetic beads for 1 h at room temperature by gentle shaking to release the **dsDNAs. Biotin-dsDNA** in the supernatant (20 μL) was bound with fresh magnetic beads as described above. After removal of supernatant, **Biotin-dsDNA** labeled magnetic beads were washed with milliQ (100 μL) and heated at 95 °C for 5 min in the fresh milliQ (50 μL). After cooling on ice (15 sec), the collected supernatant was diluted with milliQ water (×100), the sample was quantified using qPCR.

Protein quantification in the cell lysate

After the collection of medium, the intracellular protein was collected by the lysis buffer (10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.05% Triton-X). After 15 min incubation, the suspension was centrifugated at 12,000 g for 3 min at 4°C and the supernatant was used for protein quantification using Bradford assay.

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Chapter 3

Chapter 4

Development of sensing motif-tethered oligodeoxynucleotides toward multiplex bimolecular analysis.

Abstract

In this study, we developed DNA-based molecular probes which tethered the small molecule sensor motif to achieve simultaneous analysis of multi-component biomolecules. The DNA-based molecular probes gave the primary amine motif in the product DNA upon the reaction with the target biomolecule. After the labelling with the biotin at the primary amine group, the product DNA were selectively collected by the streptavidin-labelled magnetic beads to be quantified by the qPCR. In this study, we designed two types of DNA based probes to detect the glutathione (GSH) and hydrogen peroxide (H_2O_2), respectively. We succeeded in the detection of each target by biotin labelling, collection of product DNA and qPCR. Furthermore, we enabled to detect the endogenous GSH in the cell lysate.

4-1 Introduction

Various molecular probes have been developed for biomolecular analysis. Among them, fluorescent molecular probes are a powerful methodology for analyzing the dynamics of target molecules in the living cells. As a promising probe, functional fluorescence probes have been developed, which has been designed to change the characteristics of fluorescence owing to the chemical reaction with the target biomolecules.[1] For example, Maeda and co-workers have been developed the fluorescent molecular probe to monitor the thiol metabolites by employing the 2,4-dinitrobenzensulfonyl functionality, which can release the phenol group by nucleophilic thiol reactively to turn on the fluorescence.[2] Chang and co-workers reported the H_2O_2 responsive fluorescence molecular probes by using the oxidative cleavage reaction.[3] Installation of boronic ester groups at the 3' and 6' positions of a fluorescein scaffold suppressed the fluorescence signal. H_2O_2-mediated deprotection generates the strong fluorescence signal via the conversion of arylboronate to phenol.

These reaction-based fluorescent molecular probes selectively responded to the target molecules in a complex biological system. Although many sensing units have been developed to monitor the target molecules as described above, the simultaneous analysis of multi-component biomolecules using the fluorescence is limited by "color barrier" (the number of simultaneously resolvable targets is limited to four to six) owing to the overlapping the wavelength of fluorescent dye.[3] To overcome these limitations, the new type of molecular probe has been reported by using another modality such as Raman spectroscopy. For example, the super multiplexed vibrational imaging was achieved by the two kinds of Raman probes called MARS and Carbow.[4,5] However, in general, the

sensitivity of Raman spectroscopy is relatively low, which prevent the application of simultaneous analysis using the Raman spectroscopy.

Recently, the DNA encoding strategy has also been developed to detect the biological targets.[6] A variety of DNA sequences enabled to be synthesized automatically. Furthermore, the use of next-generation sequencers (NGS) made it possible to quantify a huge variety of DNA sequences with high sensitivity. In fact, it has also become possible to encode information on individual cells, enabling single cell transcriptome analysis.[7] Antibody with DNA barcode enabled the simultaneous analysis of the transcriptome and membrane surface antigens at single cell level.[8] Furthermore, target responsive DNA probes have been paid attention to expand the target biomolecules applicable to DNA encoding strategy.[9,10,11] For example, we designed the glutathione (GSH) responsive DNA probe and succeeded in the monitoring of endogenous GSH and mRNA expression by using the target responsive DNA probes labeled on the streptavidin coated magnetic beads. [10] Except for the GSH, we achieved to detect the lactate, and glucose. [11]

Herein, we designed new type of DNA-based molecular probes, which enabled to convert biomolecular information into DNA sequence information by using chemical reaction in the aqueous solution. After the reaction with the targets, we collected the product DNA by the ethanol precipitation, biotin labelling and collection using streptavidin-coated magnetic beads. In this study, we chose two biomolecules as the target, GSH from the metabolites, and H_2O_2 from the reactive oxygen species. We designed the DNA-based molecular probes by tethering the suitable small molecule sensing unit at the end of DNA strand, and succeeded in the detection of each target. Especially, we confirmed that the sensitivity of GSH detection was improved compared with the

previous method using DNA labeled magnetic beads and succeeded in the monitoring of endogenous GSH in the cell lysate.

4-2 Result & Discussion

In order to detect a variety of biomolecules, we designed the small molecular sensing unit tethered oligodeoxynucleotides, which selectively reacted with the target molecules at the end of DNA. As a proof of concept, we chose GSH, and H_2O_2 as a target molecule. GSH is an important biomolecule that regulates the redox balance *in vivo* and has been implicated in various diseases, including cancer and Alzheimer's disease.[12] H_2O_2 is known to play a central role among reactive oxygen species and is strongly correlated with oxidative stress.[13] Thus, these two targets play an important role in the cells and can be utilized as the biomarker for the disease diagnosis.

In principle, we designed functional oligodeoxynucleotides that gives a primary amine group at the end of product DNA to label with biotin as a collection tag (Figure 1a). According to the design of sensing unit in the previous reports,[14,15] we developed two DNA-based molecular probes, Mal-DNA and PB-DNA, for GSH and H_2O_2 detection, respectively (Figure 1b); As GSH responsive DNA probe, we introduced maleimide group at the end of DNA. The Michael addition reaction between thiol group of GSH and maleimide group of DNA probe gives product DNA bearing primary amine. As H_2O_2 responsive DNA probe, we tethered phenylboronic acid at the end of DNA to gives the primary amine group in the product DNA via the decarboxylation.

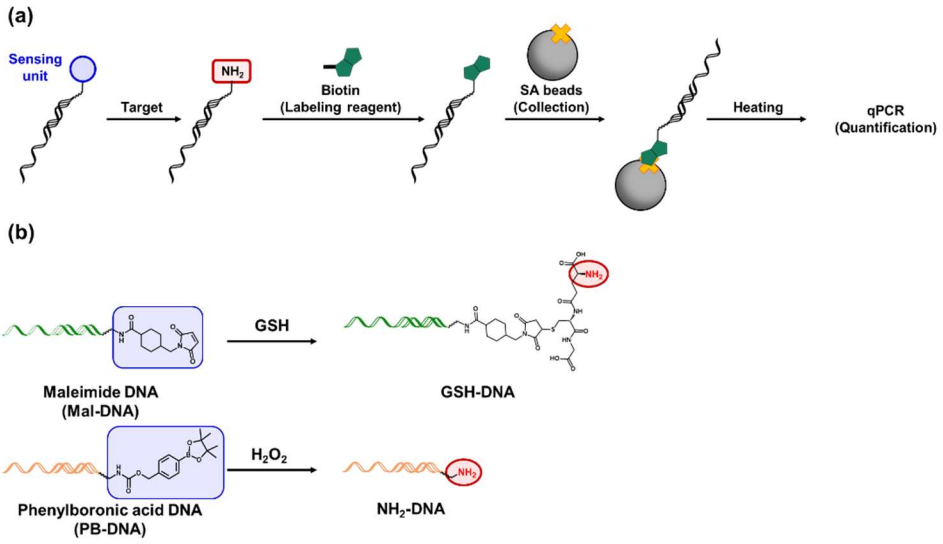

Figure 1. (a) Schematic illustration of target molecule analysis using functional oligodeoxynucleotide. (b) Reaction of Mal-dsDNA with GSH and PB-dsDNA with H_2O_2.

First, we synthesized two DNA based molecular probes: Mal-DNA was successfully synthesized by using DNA-NH_2 and sulfo-SMCC. PB-DNA was synthesized as reported previously.[11]

Next, we evaluated the reaction of DNA probes with each target biomolecules by using HPLC (Figure 2). As shown in Figure 2a, Mal-DNA reacted with GSH in phosphate buffer (pH7,.4) to give the GSH adduct oligodeoxynucleotide (GSH-DNA). The yield of GSH-DNA increased in a GSH dose-dependent manner (Figure 2b). For PB-DNA, it was also confirmed that new product peaks were obtained in the presence of the target and that the reaction proceeded in a H_2O_2 dose dependent manner (Figure 2c, d). These data represent that the DNA probes reacted with the target and gave the product DNA selectively.

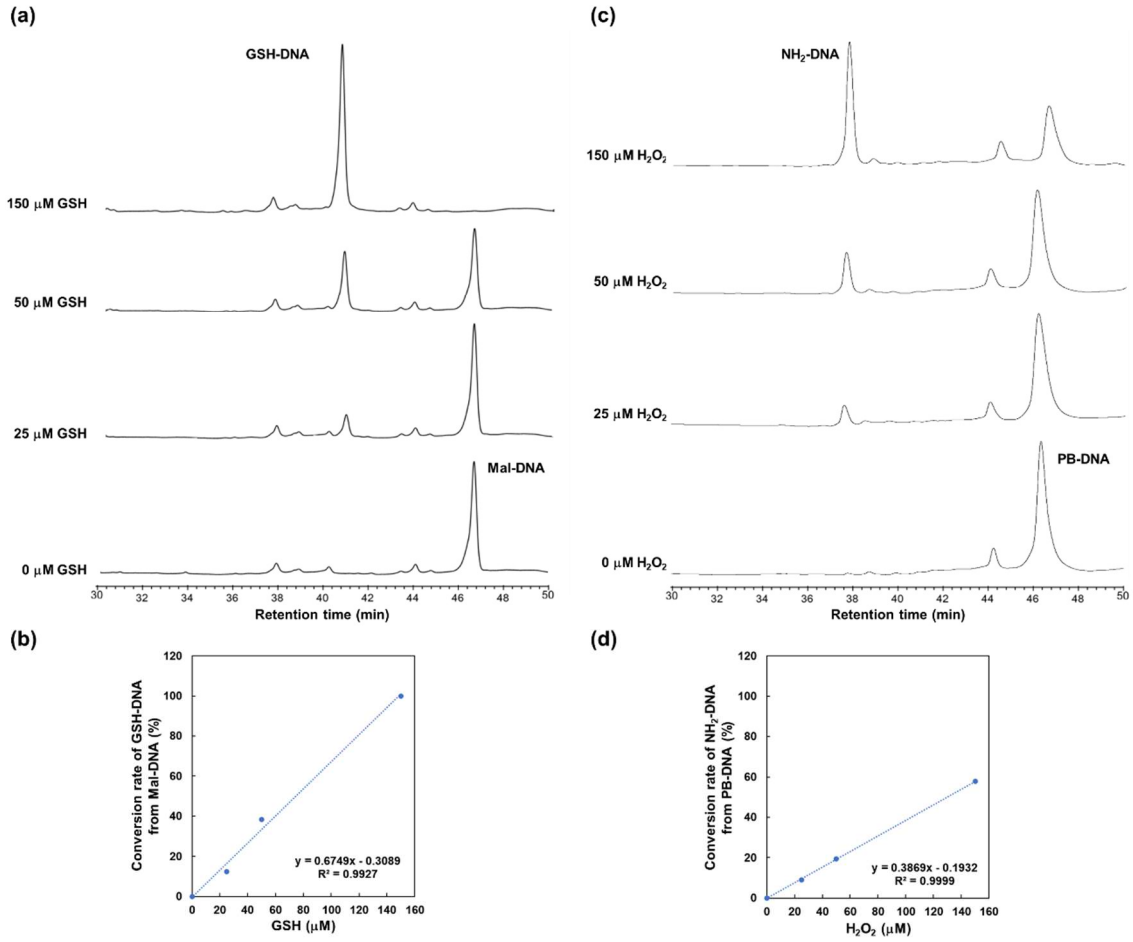

Figure 2. (a) HPLC chart of 5 μM Mal-DNA incubated with 0, 25, 50, or 150 μM GSH in PB (250 mM pH7.4) for 1 h at r.t. (b) Conversion rate of GSH-DNA from Mal-DNA. (c) HPLC chart of 5 μM PB-DNA incubated with 0, 25, 50 or 150 μM H_2O_2 in PB (250 mM pH7.4) for 1 h at r.t. (d) Conversion rate of NH_2-DNA from PB-DNA.

Having the suitable DNA probes for GSH and H_2O_2 in hand, we prepared duplex DNA (NH_2-dsDNA) consisted of NH_2-DNA as product DNA and 60-mer code DNA and tested the ability to collect the dsDNA-NH_2 via following steps; 1st step: biotin labelling, 2nd step: ethanol precipitation, 3rd step: collection using streptavidin-coated magnetic beads, 4th step: releasing the code DNA and quantification (Figure 3). The protocol for recovery and quantification of code DNA was as follows (Figure 3a). First, NH_2-dsDNA was biotinylated by *N*-hydroxysulfosuccinimide ester tethered biotin (Biotin-AC_5 sulfo-OSu), followed by ethanol precipitation to remove unreacted biotin-AC_5 sulfo-OSu.

The biotinylated DNA was collected using SA beads and then, the code DNA was released in supernatant by the heating to be quantified by the qPCR (Figure 3b). As a result, after incubation of NH_2-dsDNA with biotin-AC_5 sulfo-OSu and following collection, a significant amount of code DNA was recovered. On the other hand, without treatment with biotin-AC_5 sulfo-OSu, negligible amount of code DNA was obtained. In a separate experiment, a similar reaction was performed with pre-biotinylated ODN (Biotin-dsDNA) to evaluate the effect of biotinylation. Similar to the reaction of NH_2-dsDNA with biotin-AC_5 sulfo-OSu, similar amount of code DNA was recovered, indicating that NH_2-dsDNA was biotinylated completely and that unreacted biotin-AC_5 sulfo-OSu was removed by ethanol precipitation. Thus, biotinylation and following collection of labeled DNAs can be applied to sensing of target biomolecules.

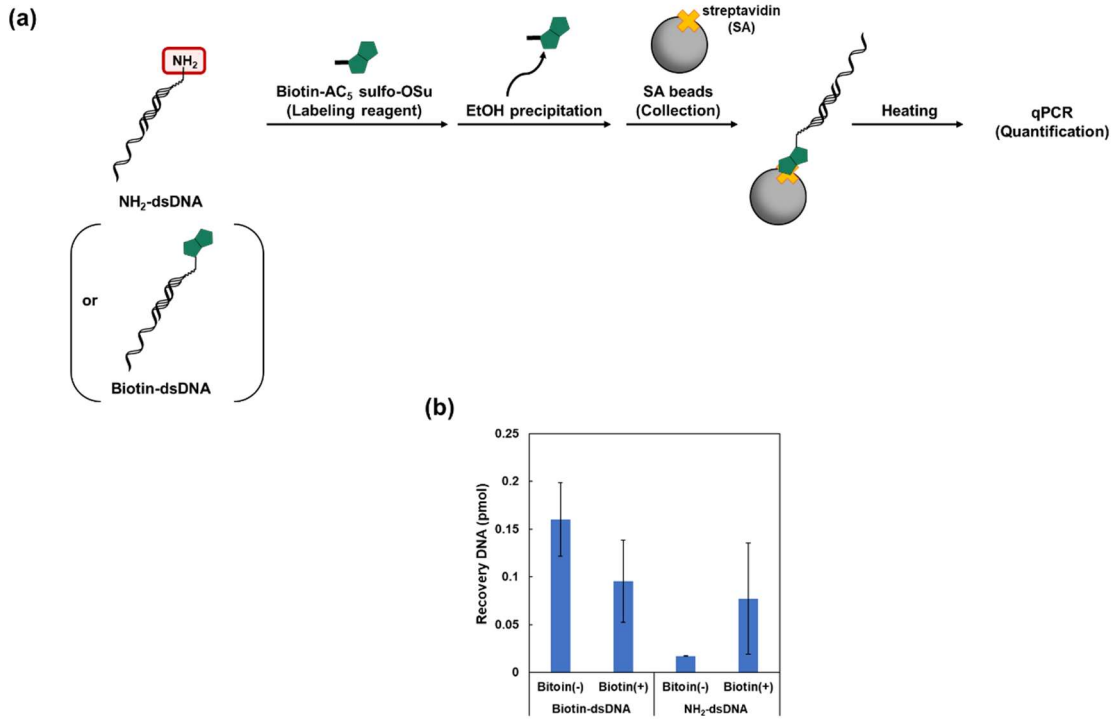

Figure 3 (a) Schematic illustration of collection of NH_2-dsDNA using biotin labeling and streptavidin magnetic beads. (b) Quantification of collected DNA using 1.5 pmol biotin-dsDNA or NH_2-dsDNA after incubating with 0 or 100 nmol biotin-AC_5 sulfo-OSu in carbonate-bicarbonate buffer (100 mM, pH8.7) for 1 h at r.t. Data = mean ± S.D. (n = 3)

Further attempts were made to detect the target molecules by using the present DNA encoding methods (Figure 4a). First, we verified whether the two DNA probes could detect each target biomolecules. The DNA probe was reacted with or without the target for 1 h and then then collected the code DNA via biotin-labeling. As shown in Figure 4b, the amount of code DNA increased in the presence of their target molecules, indicating that the present system worked as expected. Next, we evaluated the detection limit of GSH using the Mal-dsDNA, GSH responsive DNA probe (Figure 4c). The measurement of the amount of code DNA revealed that 4 pmol of GSH in the sample could be detected by the present DNA encoding method. Thus, GSH has been successfully encoded in DNA, and we succeeded in improving the sensitivity approximately 100 folds by using present method.

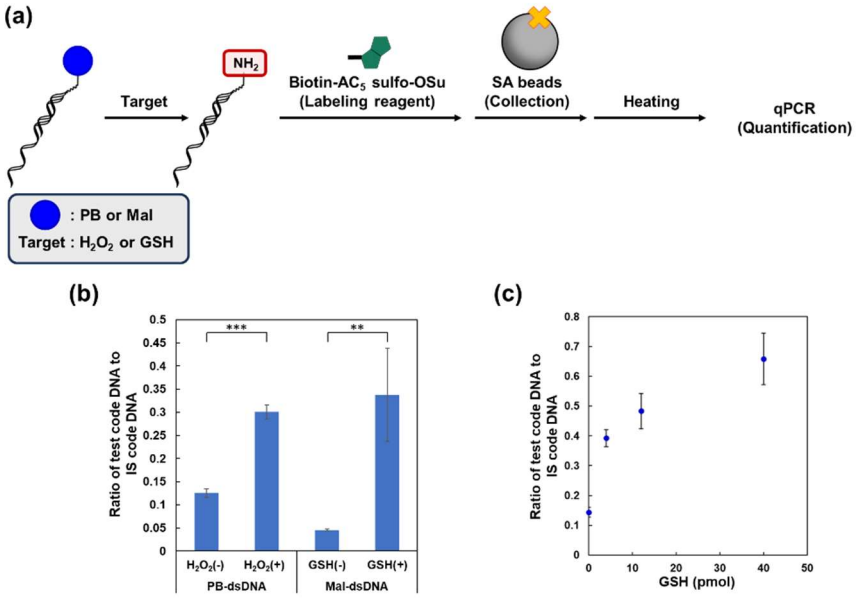

Figure 4. (a) Schematic illustration of quantification of H_2O_2 and GSH using PB-dsDNA and Mal-dsDNA. To normalize the amount of collected code DNA, we utilized biotin-dsDNA with different sequences as the internal standard. The recovery of DNA was corrected using the amount of code DNA. (b) Detection of 0 or 10 nmol H_2O_2 using 1.5 pmol PB-dsDNA and 0 or 10 nmol GSH using 1.5 pmol Mal-dsDNA. (c) Detection of 0 or 4 pmol GSH using 1.5 pmol dsDNA. Data = mean ± S.D. (n = 3, ** indicates $p<0.05$, *** indicates $p<0.01$)

Finally, we tested whether this DNA encoding method could be used to detect endogenous GSH in the cell lysate (Figure 5). Human lung cancer cell line, A549 cell lysates were prepared, and DNA probes were reacted in the cell lysate with and without excess of *N*-Methylmaleimide (NMM) (Figure 5a). NMM acts as a thiol masking reagent in cell lysate. As shown in Figure 5b, a huge of amount of code DNA was recovered from the sample without treatment by NMM, whereas recovery of code DNA was suppressed from the sample treated by an excess amount of NMM. Thus, the selective detection of thiol metabolites, mainly GSH, in the cells was achieved by using present procedure.

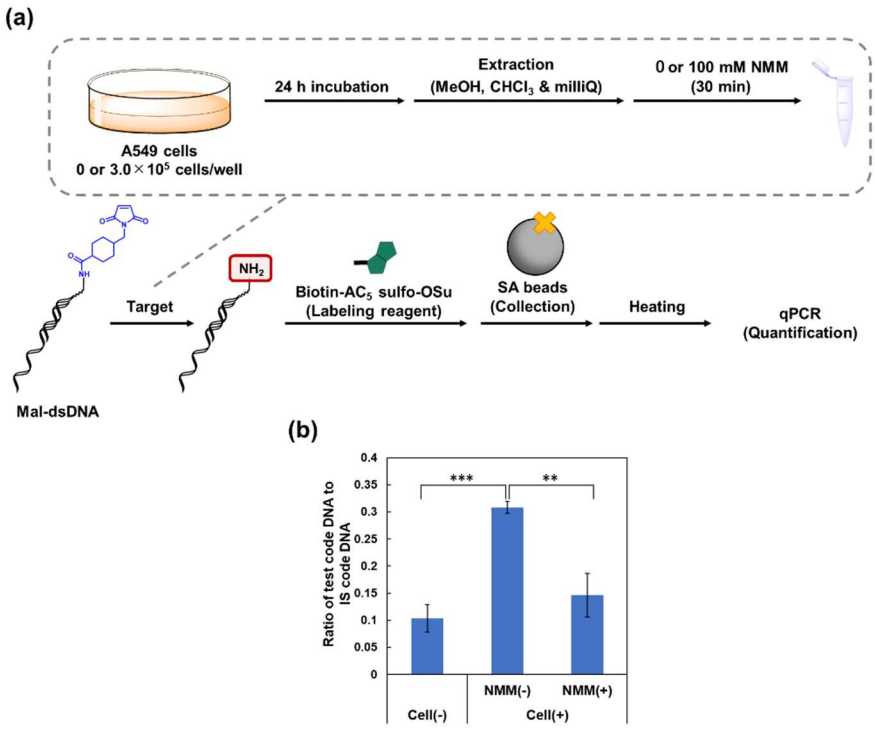

Figure 5. (a) Schematic illustration of detection of GSH derived from cell extract after reacting with 0 or 100 mM NMM. (b) Detection of thiol metabolites in cell extract using 1.5 pmol Mal-DNA. Data = mean ± SD (n = 3, ** indicates $p<0.05$, *** indicates $p<0.01$)

4-3 Conclusion

In summary, we developed functional oligodeoxynucleotides equipped with sensing sites for target molecule to analyze multi-component biomolecules. We found that the information of GSH and H_2O_2, important molecular species in living cells, can be converted into the DNA sequence information by using chemical reactions. We constructed a molecular system that primary amine group tethered DNA was given by reacting with target molecule and the biotin labeling of amine groups enables detection of target molecule by quantification of biotinylated DNA. This methodology can be widely applied not only to the analysis of the present two targets but also that of other

biomolecules, especially enzymes. Moreover, it is expected that the method will be used in combination with next-generation sequencers to realize the analysis of super-multicomponent biomolecules in the future.

4-4 Experimental section

General Methods

Reagents were purchased from Wako pure chemical industries, Sigma Aldrich, Nacalai Tesque, Takara Bio, Dojindo, Tokyo chemical industries, New England BioLabs, and eurofins Genomics. Analytical and preparative high-performance liquid chromatographies were performed on a D2000 HPLC system and L7000 HPLC system (HITACHI) with reversed phase column (InerrtsilvODS-3, GL Science Inc., ϕ 1.0 mm×250 mm (particle size; 5 μm), ϕ 4.6 mm×250 mm (particle size; 5 μm)). MALDI-TOF mass spectrometry were recorded on an AXIMER-LNR spectrometer (SHIMADZU). DNA hybridization and quantitative PCR (qPCR) analysis was carried out with MyGo Mini S Real Time PCR (IT-IS Life Science Ltd.). DNA sequences are shown in Table 1.

Table 1. Sequences in this study

Strand name	Sequences (5' to 3')
NH_2-DNA (test)	GGT CCA ATC TAC AGG AAT TC-NH_2
Test code ODN	GAA TTC CTG TAG ATT GGA CCT TTT TTT TTT TTT TTT TTT CGA CCC TAA GCA TAC ATA CCT
Forward primer	AGG TAT GTA TGC TTA GGG TCG
Reverse primer	GAA TTC CTG TAG ATT GGA CC
NH_2-DNA (internal standard)	CTT AAG GAC ATC TAA CCT GG-NH_2
Internal standard code DNA	CCA GGT TAG ATG TCC TTA AGT TTT TTT TTT TTT TTT TTT TCC ATA CAT ACG AAT CCC AGC
Forward primer (for IS code DNA)	GCT GGG ATT CGT ATG TAT GGA
Reverse primer (for IS code ODN)	CCA GGT TAG ATG TCC TTA AG

Synthesis of Mal-DNA

The solution of sulfo-SMCC (100 mM in milliQ, 2.0 μmol) were added the solution of NH_2-DNA (1 mM in milliQ, 5.0 nmol) in 100 mM pH8.7 carbonate-bicarbonate buffer and incubated at room temperature for 5 min. The reaction mixture was purified using Micro Bio-Spin™ 6 Columns (Bio-Rad) and milliQ water as an elution solution. The concentration of the oligomer was determined by complete digestion with alkaline phosphatase (AP), nuclease P1 (PI) and phosphodiesterase I at 37°C for overnight. Identities of synthesized oligomers were confirmed by MALDI-TOF MS spectrometry ($[M-H]^-$ calcd. 6525.22, found. 6524.51). The purity of synthesized oligomer was confirmed by monitoring of UV absorbance at 260 nm at a flow rate of 0.6 (analysis) $mLmin^{-1}$ with a 0–25% (over 50 min) gradient of acetonitrile (ACN) / triethylammonium acetate buffer (TEAA buffer) (100 mM, pH 7.0).

Synthesis of PB-DNA

The solution of phenylboronic acid N-hydroxysuccinimide ester (500 mM in DMSO, 10 μmol) were added the solution of NH_2-DNA (1 mM in milliQ, 5 nmol) in 100 mM pH8.7 carbonate-bicarbonate buffer containing 50% DMSO and incubated at room temperature for 2 h. The reaction mixture was purified using Micro Bio-Spin™ 6 Columns (Bio-Rad) and milliQ water as an elution solution. The concentration of the oligomer was determined by complete digestion with alkaline phosphatase (AP), nuclease P1 (PI) and phosphodiesterase I at 37°C for overnight. Identities of synthesized oligomers were confirmed by MALDI-TOF MS spectrometry using saturated 3-hydroxypicolinic acid in 100 mM diammonium hydrogen citrate solution as a matrix ($[M-H]^-$ calcd. 6566.26, found. 6566.03). The purity of synthesized oligomer was confirmed by monitoring of UV

absorbance at 260 nm at a flow rate of 0.6 (analysis) $mLmin^{-1}$ with a 0–25% (over 50 min) gradient of acetonitrile (ACN) / triethylammonium acetate buffer (TEAA buffer) (100 mM, pH 7.0).

Synthesis of Biotin-DNA

17 μL of carbonate-bicarbonate buffer (706 mM pH 8.7) containing NH_2-DNA (5.0 nmol) was added to 100 μL of Biotin-AC_5 sulfo-OSu (Dojindo) (2.2 μmol) in milliQ water. The resulting solution was kept at room temperature for overnight and was purified Micro Bio-Spin™ 6 Columns (Bio-Rad) and milliQ water as an elution solution. Moreover, the sample was purified by HPLC using ODS-3 column (ϕ 4.6 mm×250 mm (particle size; 5 μm)). The concentration of the oligomer was determined by complete digestion with AP, PI and phosphodiesterase I at 37°C for overnight. Identities of synthesized oligomers were confirmed by MALDI-TOF MS spectrometry ((For sample) $[M-H]^-$ calcd. 6645.29, found 6645.31, (For internal standard) $[M-H]^-$ calcd. 6645.29, found 6645.50,).

Reaction monitoring of PB-DNA

5 μM PB-DNA was reacted with 0, 25, 50, or 150 μM H_2O_2 in PB (250 mM, pH 7.4) for 1 h at room temperature. The reaction mixtures were analyzed by HPLC.

Reaction monitoring of Mal-DNA

5 μM Mal-DNA was reacted with 0, 25, 50, 150 μM GSH in PB (250 mM, pH 7.4) for 1 h at room temperature. The reaction mixtures were analyzed by HPLC.

DNA hybridization

500 nM code DNA, and biotin-DNA (for biotin-dsDNA) or PB-DNA (for PB-dsDNA) or Mal-DNA (for Mal-dsDNA) or NH_2-DNA (for NH_2-dsDNA) were hybridized in 5 mM $MgCl_2$. DNA was heated to 95°C for 5 min and then cooled at a rate of 1°C per min to 25°C.

Detection of biotin-dsDNA or NH_2-dsDNA using magnetic beads

75 nM biotin-dsDNA or NH_2-dsDNA were incubated with 5 mM biotin-AC_5 sulfo-OSu in carbonate-bicarbonate buffer (200 mM, pH8.7) for 1 h at room temperature. After reaction, the reacted solution was mixed with EtOH (60 μL) and sodium acetate buffer (AcONa buffer) (5 M, pH5.2, 6.5 μL), and centrifuged at 15000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with binding buffer and streptavidin coated magnetic beads (50 μg) and rotating for 10 min at room temperature. After removal of supernatant, Biotin-dsDNA labeled magnetic beads were washed with milliQ (100 μL) and heated at 95°C for 5 min in the fresh milliQ (50 μL). After cooling on ice (15 sec), the collected supernatant was diluted with milliQ water (×1000), the sample was quantified using qPCR. qPCR was performed according to the manufacturer's procedure using Luna Universal qPCR Master Mix (NEW ENGLAND Bio Labs): 2 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C.

Evaluation of an amount of biotin-AC_5 sulfo-OSu and internal standard

75 nM NH_2-dsDNA and biotin-dsDNA (IS) was incubated with 0, 0.15, 0.5, 1.5, 5 mM biotin-AC_5 sulfo-OSu (0, 3.0, 10, 30, 100 nmol) in carbonate-bicarbonate buffer (200 mM, pH8.7) for 1 h at room temperature. After reaction, the reacted solution was mixed with EtOH (60 μL) and AcONa buffer (5 M, pH5.2, 6.5 μL), and centrifuged at 15000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with binding buffer and streptavidin coated magnetic beads (50 μg) and rotating for 10 min at room temperature. After removal of supernatant, Biotin-dsDNA labeled magnetic beads were washed with milliQ (100 μL) and heated at 95°C for 5 min in the fresh milliQ (50 μL). After cooling on ice (15 sec), the collected supernatant was diluted with milliQ water (×1000), the sample was quantified using qPCR.

Detection of GSH or H_2O_2 using PB-dsDNA

75 nM Mal-dsDNA (test) or PB-dsDNA (test) and biotin-dsDNA (IS) was incubated with 0 or 500 μM GSH or H_2O_2 (0 or 10 nmol) in PB (250 mM, pH7.4) for 1 h at room temperature. After reaction, the reacted solution was mixed with EtOH (60 μL) and AcONa buffer (5 M, pH5.2, 6.5 μL), and centrifuged at 15000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with 500 μM biotin-AC_5 sulfo-OSu (10 nmol) in carbonate-bicarbonate buffer (200 mM, pH8.7) and incubated for 1 h at room temperature. After reaction, the reacted solution was mixed with EtOH (60 μL)

and AcONa buffer (5 M, pH5.2, 6.5 μL), and centrifuged at 15000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15000 g for 4 min at °C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with binding buffer and streptavidin coated magnetic beads (50 μg) and rotating for 10 min at room temperature. After removal of supernatant, Biotin-dsDNA labeled magnetic beads were washed with milliQ (100 μL) and heated at 95°C for 5 min in the fresh milliQ (50 μL). After cooling on ice (15 sec), the collected supernatant was diluted with milliQ water (×1000), the sample was quantified using qPCR.

Quantification of GSH using Mal-dsDNA

75 nM Mal-dsDNA (test) and biotin-dsDNA (IS) was incubated with 0, 0.2, 0.6 or 2.0 μM GSH (0, 4, 12, or 40 pmol) in PB (250 mM, pH7.4) for 1 h at room temperature. After reaction, the reacted solution was mixed with EtOH (60 μL) and AcONa buffer (5 M, pH5.2, 6.5 μL), and centrifuged at 15000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with 500 μM biotin-AC_5 sulfo-OSu (10 nmol) in carbonate-bicarbonate buffer (200 mM, pH8.7) and incubated for 1 h at room temperature. After reaction, the reacted solution was mixed with EtOH (60 μL) and AcONa buffer (5 M, pH5.2, 6.5 μL), and centrifuged at 15000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with binding buffer and

streptavidin coated magnetic beads (50 μg) and rotating for 10 min at room temperature. After removal of supernatant, Biotin-dsDNA labeled magnetic beads were washed with milliQ (100 μL) and heated at 95°C for 5 min in the fresh milliQ (50 μL). After cooling on ice (15 sec), the collected supernatant was diluted with milliQ water (×1000), the sample was quantified using qPCR.

Cell culture

Human lung cancer cell line, A549 were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS). The cells were maintained at 37°C in 5% CO_2 / 95% air and were kept in a logarithmic growth phase by routine passages every 2−3 days. Prior to the use of cells, the densities of cells were determined using a hemocytometer.

Detection of GSH derived from cell extract

A549 cells (0 or 3.0×10^5 cells per well) were seeded onto 6 well plate in 3 mL DMEM. Plate was maintained at 37°C in 5% CO_2 / 95% air incubator for 24 h. After washing with D-PBS (-) twice, methanol (500 μL) was added into each well and incubated for 15 min. Then, cell solution (200 μL) were collected and extracted with 100 μL milliQ and 200 μL of $CHCl_3$. After centrifugation at 12,000 g for 15 min at 4°C, aqueous layer (250 μL) was lyophilized. Cell extract was dissolved with 0 or 100 mM N-methylmaleimide (12 μL) and incubated for 30 min.

75 nM Mal-dsDNA and biotin-dsDNA (IS) was incubated with cell extract (10 μL) and PB (500 mM, pH7.4, 10 μL) for 1 h at room temperature. After reaction, the reacted solution was mixed with EtOH (60 μL) and AcONa buffer (5 M, pH5.2, 6.5 μL), and

centrifuged at 15000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with 500 μM biotin-AC_5 sulfo-OSu (10 nmol) in carbonate-bicarbonate buffer (200 mM, pH8.7) and incubated for 1 h at room temperature. After reaction, the reacted solution was mixed with EtOH (60 μL) and AcONa buffer (5 M, pH5.2, 6.5 μL), and centrifuged at 15000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with binding buffer and streptavidin coated magnetic beads (50 μg) and rotating for 10 min at room temperature. After removal of supernatant, Biotin-dsDNA labeled magnetic beads were washed with milliQ (100 μL) and heated at 95°C for 5 min in the fresh milliQ (50 μL). After cooling on ice (15 sec), the collected supernatant was diluted with milliQ water (×1000), the sample was quantified using qPCR.

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Chapter 5

Target cell specific cytotoxicity using streptavidin scaffold bearing cell recognition oligonucleotide and photoactivated drug

Abstract

Prodrugs that present strong cytotoxicity toward specific cells have been utilized for cell-type selection and purification. In this study, we designed and prepared a multifunctional, photoactivated prodrug based on a streptavidin scaffold. Biotin-labeled DNA aptamer that recognizes the membrane antigen EpCAM, and biotin-labeled photoactivated prodrug bearing the antitumor camptothecin, were prepared. Both molecules were linked to the streptavidin scaffold by simple mixing. The resulting prodrug bound to the EpCAM-overexpressing SK-BR-3 target cells and showed cytotoxic effects upon photoirradiation, corresponding to cytotoxic drug (camptothecin) release.

5-1 Introduction

Attempts to use prodrugs for cell-type selection and purification have made progress in recent years.[1–4] In particular, prodrugs are used to remove undifferentiated human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) and enable cell-type purification without a cell sorter. Prodrugs play important roles in disabling undesired cells for regenerative medicine.

In many cases, prodrugs have been activated by cell-specific reactive molecules such as enzymes to induce cell toxicity.[1] For example, phospho-D-peptide was reported as a hiPSC-eliminating agent, which was activated by the alkaline phosphatase overexpressed on the hiPSC cell surface. After dephosphorylation, hydrophobic peptide aggregated and induced cell death. Another strategy is to utilize a ligand that recognizes the target cells to deliver the drug. For example, rBC2LCN (recombinant N-terminal domain of BC2L-C lectin derived from *Burkholderia cenocepacia*) was used as a ligand to eliminate human pluripotent stem cells (hPSCs).[2] The rBC2LCN bearing the toxin can eliminate undifferentiated cells from the differentiated cell population, lowering the risk of teratoma formation during treatment with hPSC-based regenerative medicine.[3] More recently, a combination of rBC2LCN and photochemical approaches was applied to eliminate undifferentiated hPSCs.[4] Although these approaches are useful, it is still difficult to specifically control the function of living cells, and finding the optimal trigger to express cytotoxicity that is specific to target cells remains a challenge.

In this study, we designed a novel prodrug consisting of a streptavidin scaffold bearing a photoactivated prodrug and an oligonucleotide aptamer. Among various recognition units, we selected oligonucleotide aptamers for cell recognition.

Oligonucleotides (ODNs) can be synthesized by using well-established automated solid-phase synthesis in a simple, rapid, and cost-effective manner. In addition, SELEX methodology[5,6] can be used to generate several aptamers, which bind with not only the target antigen on the cell surface but also, in principle, to target cells themselves. To construct a multifunctional prodrug, streptavidin (SA) was employed in this study. Streptavidin is known to readily bind four biotin molecules by simply mixing. In light of this character, we expected that streptavidin could act as a scaffold for functionalization with biotin-labeled molecules including ODNs and photoactivated prodrugs. As shown in Figure 1, we prepared photoactivated prodrug **SA–Camp-PC-biotin–DNA complex**. This molecule consisted of two units, biotin labeled DNA aptamer (DNA complex), and photocleavable prodrug (**Camp-PC-biotin**), which were connected through a streptavidin scaffold. The DNA aptamer recognizes EpCAM on the cell surface,[7] whereas the prodrug unit releases the antitumor agent camptothecin upon activation through photoirradiation at 365 nm. We envisioned that the **SA–Camp-PC-biotin–DNA complex** would bind selectively to target cells expressing EpCAM and that photoirradiation would lead to release of camptothecin and to damage of the target cells. Here, we demonstrate a proof-of-concept study using **SA–Camp-PC-biotin–DNA complex** as a prodrug. The complex was easily prepared, and it showed strong cytotoxic effect against EpCAM-expressing cells upon photoirradiation.

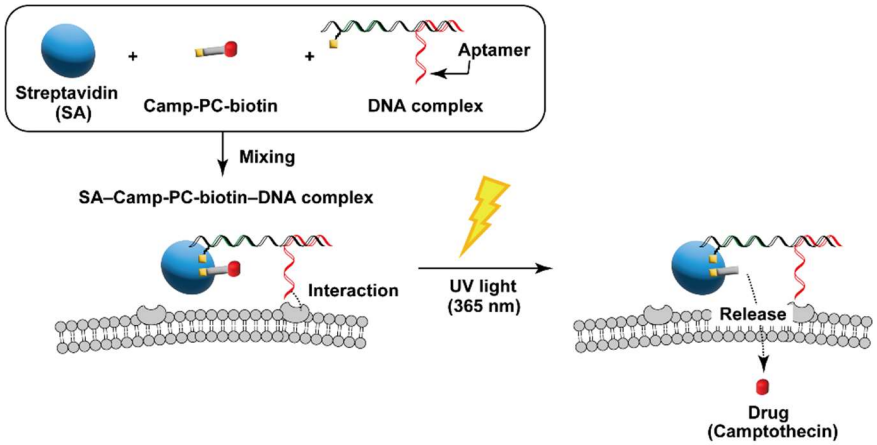


Figure 1. Schematic illustration of multifunctional photoactivated prodrug on a streptavidin scaffold bearing a DNA aptamer.

5-2 Results and Discussion

Initially, we prepared the streptavidin–DNA complex (**SA–DNA complex**). ODNs bearing both biotin and FAM units (**biotin strand-FAM**) and an aptamer bearing Cy5 units (**recognition strand-Cy5**) were hybridized with **template strand** to form a **DNA complex**. The **DNA complex** was then bound with streptavidin (Figure 2a) to give the **SA–DNA complex**. Binding of streptavidin to the **DNA complex** was confirmed using agarose gel electrophoresis (Figure 3). We also prepared **SA–DNA complex APT(–)**, without the DNA aptamer unit, as a control complex.

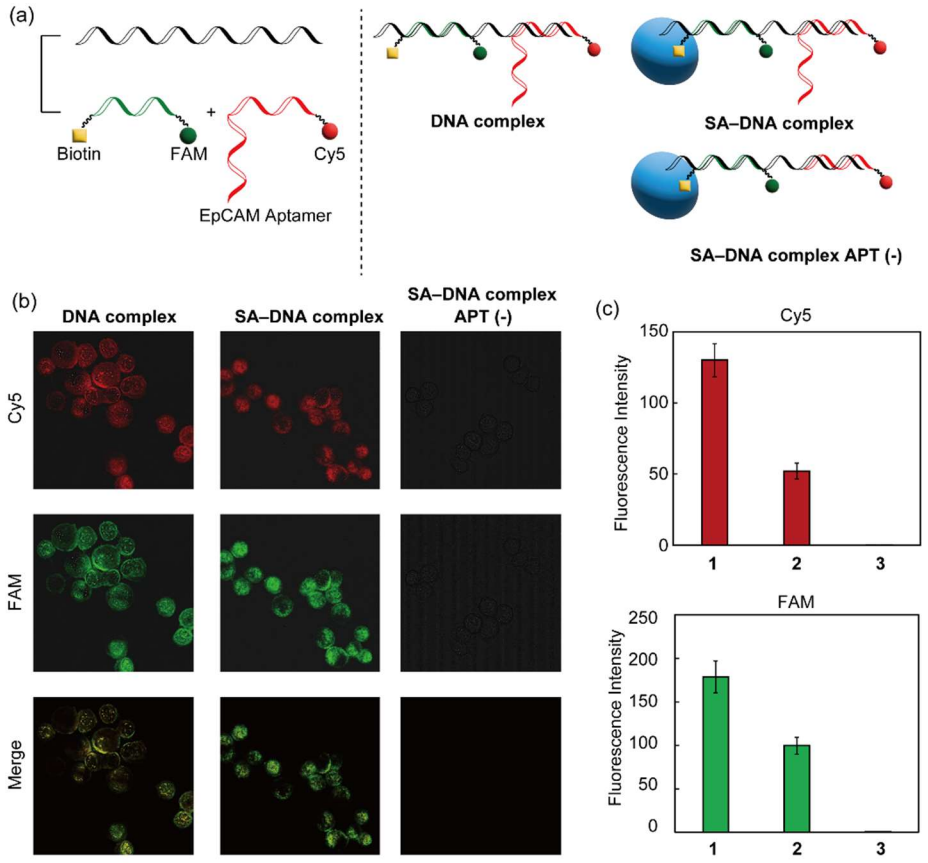


Figure 2. (a) Recognition materials used in this study to recognize the target cell, SK-BR-3 (b) Fluorescence emission from **DNA complex**, **SA-DNA complex,** and **SA-DNA complex APT (-)** (FAM: Ex. = 488 nm, Cy5: Ex. = 640 nm). SK-BR-3 cells ware incubated with each recognition material (200 nM) in D-PBS (5 mM $MgCl_2$) for 10 min at 4°C (c) The relative emission intensity from the cells incubated with each recognition materials (Data = mean ± SE, n = 19) **1**: **DNA complex**, **2**: **SA–DNA complex**, **3**. **SA–DNA complex APT (-)**.

We then conducted cellular experiments with the **DNA complex** and **SA–DNA complex** using human breast adenocarcinoma (SK-BR-3) as a model target cell that overexpressed EpCAM.[8] SK-BR-3 cells were incubated with either the **DNA complex** or the **SA–DNA complex**, and the fluorescence emission of FAM and Cy5 on the complex was imaged with a confocal laser microscope. As shown in Figure 2b, emissions of both FAM and Cy5 were observed from the cells that were incubated with these complexes. Thus,

efficient cellular uptake was found for both complexes. On the other hand, cells incubated with the **DNA complex** showed stronger emission than cells incubated with the **SA–DNA complex** (Figure 2c), indicating that the affinity of the DNA aptamer to EpCAM may be weakened by steric hindrance of streptavidin. When **SA–DNA complex Apt(–)** was incubated with SK-BR-3 cells, negligible emissions were observed from the cells (Figure 2b, Table S1). These results indicate that the DNA aptamer unit is indispensable for efficient cellular uptake. Thus, the **SA–DNA complex** is suitable for delivering the prodrug to the target cells.

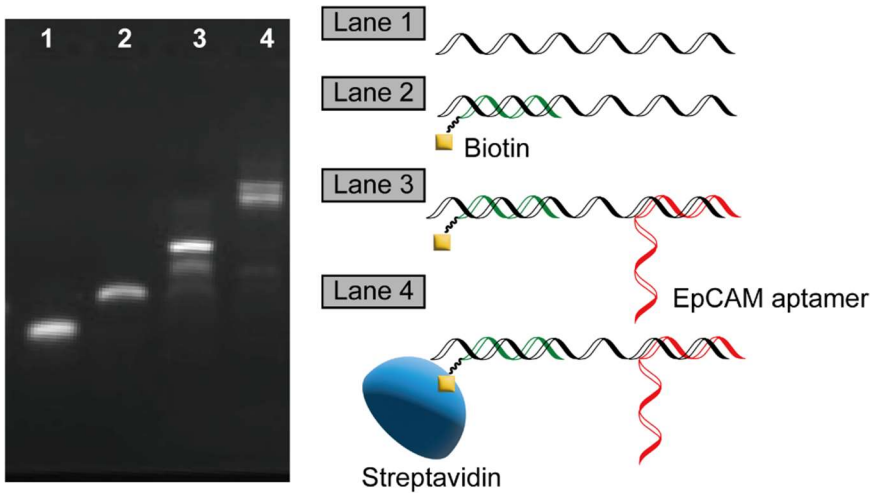
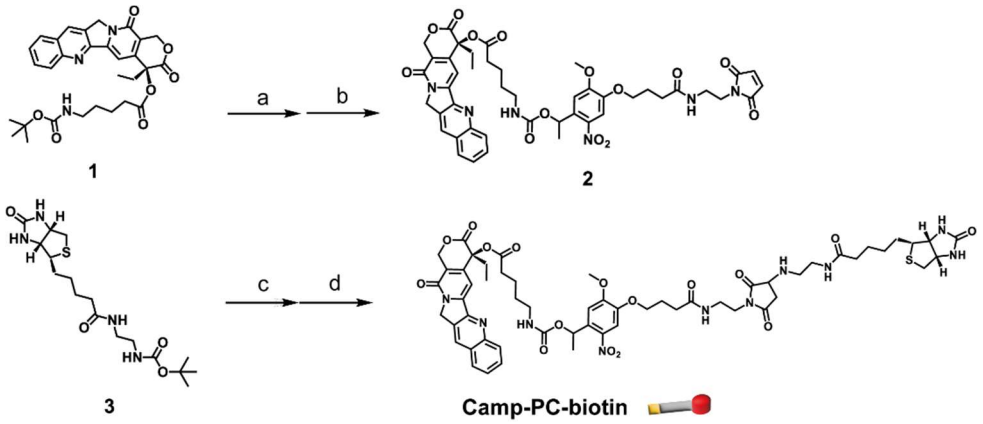


Figure 3. Evaluation of streptavidin-DNA complex (**SA–DNA complex**) formation. 3.5% agarose gel electrophoresis (Lane 1. 10 pmol template strand, Sample 2. 10 pmol template strand + 10 pmol biotin strand, Lane 3. **DNA complex** (10 pmol template strand + 10 pmol biotin strand + 10 pmol recognition strand), Lane 4. 10 pmol **DNA complex** + 10 pmol streptavidin.

Next, we designed and constructed the biotin-tethered photocleavable prodrug (**Camp-PC-biotin**). We selected camptothecin as a model drug, which is known to be an inhibitor of topoisomerase.[9] Among the triggers that are available for activation of prodrugs,[10–12] photoirradiation was selected because of the high spatiotemporal resolution that can be reached by using the technique. We introduced a photoresponsive *ortho*-nitrobenzyl group and a biotin unit on camptothecin at the 20-position to prepare **Camp-**

PC-biotin, which can bind to streptavidin.[13] The synthesis of **Camp-PC-biotin** is outlined in Scheme 1. Camptothecin derivative **1** was treated with TFA to remove the BOC group, then the nitrobenzyl unit[14] was introduced to form **2**. The latter was finally coupled with the biotin unit to give **Camp-PC-biotin**.



Scheme 1. Regents and conditions. (a) TFA, DCM, r.t. (b) Photocleavable linker, Et_3N, DMF, r.t. 17%. (c) TFA, DCM, r.t. (d) **2**, DMF r.t. 54%

We verified the release of the camptothecin from **Camp-PC-biotin** upon photoirradiation. As shown in Figure 3a, HPLC analysis of the reaction confirmed that the *ortho*-nitrobenzyl moiety of **Camp-PC-biotin** produced **Camp-NH$_2$** upon photoirradiation at 365 nm. Rapid disappearance of **Camp-PC-biotin** (retention time: 52 min) was observed after photoirradiation for 15 min to form **Camp-NH$_2$** (32 min). The latter slowly converted into camptothecin (38 min). After 72 h incubation, 24% of camptothecin was released from the **Camp-PC-biotin** (Figure 3c).

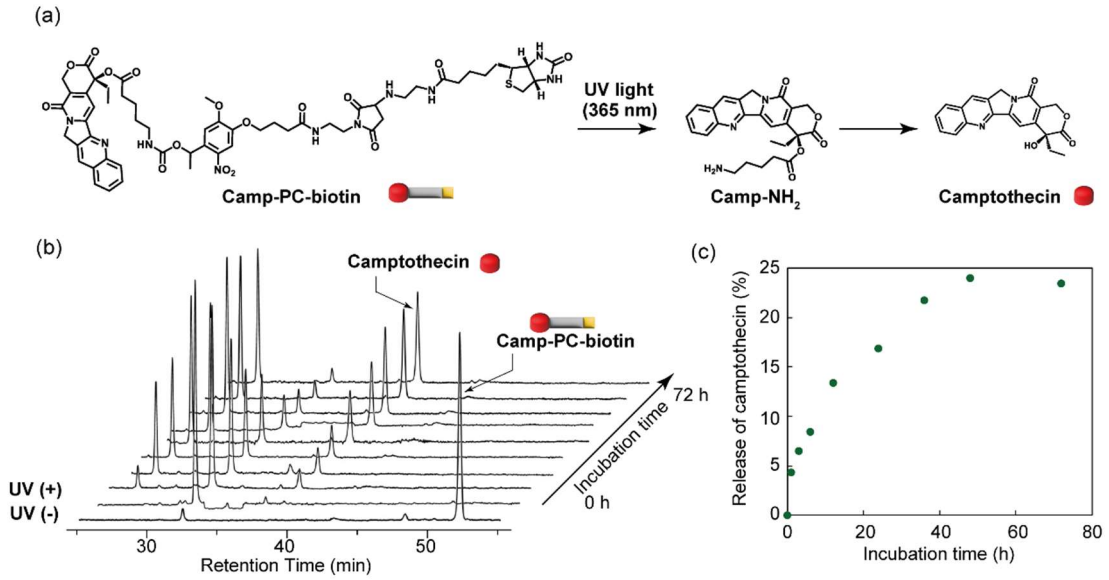


Figure 3. (a) Chemical conversion from **Camp-PC-biotin** to camptothecin upon the photo irradiation (365 nm) (b) Reaction monitoring using the HPLC. 10 μM **Camp-PC-biotin** in 10 mM PB (pH 7.4, 10% DMSO) for 0 or 15 min photo irradiation (365 nm). (c) Conversion ratio from **Camp-PC-biotin** to camptothecin after photo irradiation (incubation time: 0–72 h).

To evaluate photoinduced release of camptothecin from **Camp-PC-biotin** and **SA–Camp-PC-biotin** to induce cytotoxic effects, we conducted cell viability assays using these molecules. To characterize their toxic effects, SK-BR-3 cells were irradiated in the presence of either **Camp-PC-biotin** or **SA–Camp-PC-biotin** for 0 or 15 min at 365 nm. After 72 h incubation, the cells were subjected to WST assay. As shown in Figure 4a, b, **Camp-PC-biotin** and **SA–Camp-PC-biotin** showed little toxicity up to a concentration of 150 nM under non-photoirradiation conditions. In contrast, photoirradiation of the cells in the presence of either of the molecules resulted in significant toxicity, indicating that photoirradiation led to the release of camptothecin from these molecules and to almost same cytotoxicity levels. To confirm the drug release, we conducted the photoirradiation of **SA–Camp-PC-biotin**. Native PAGE analysis revealed that fluorescence of camptothecin was observed in the band attributed to streptavidin before photoirradiation,

whereas the fluorescence in the band disappeared after irradiation (Figure 4). Thus, camptothecin was released from streptavidin upon photoirradiation and induced toxic effects.

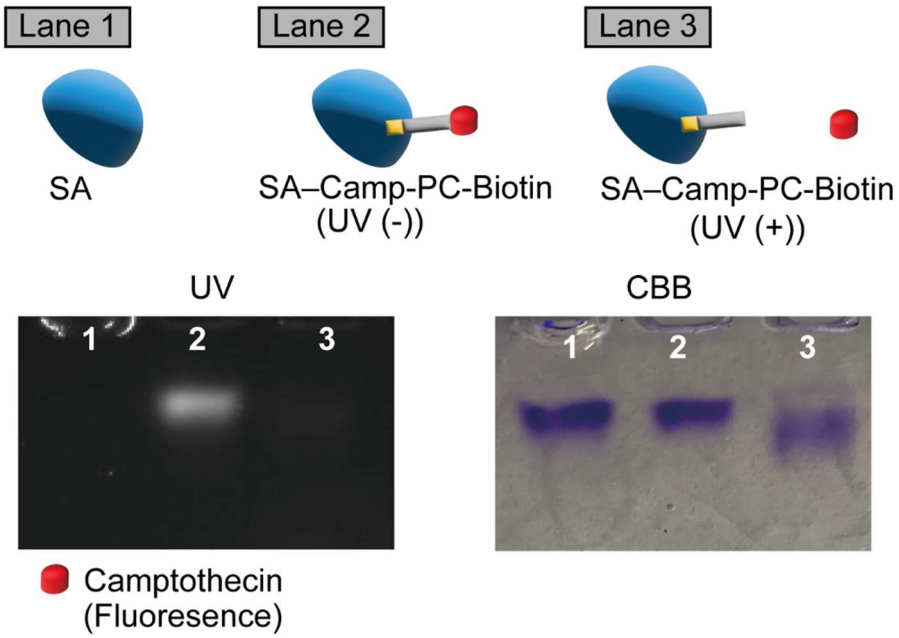


Figure 4. Drug release from streptavidin upon the photo irradiation. 10% native-PAGE analysis (Lane 1. 50 pmol streptavidin, Lane 2. 50 pmol streptavidin and 150 pmol Camp-PC-Biotin incubated for 30 min at ambient temperature, Lane 3. 50 pmol streptavidin and 150 pmol Camp-PC-Biotin incubated for 30 min at ambient temperature and irradiated at UV light (365 nm) for 15 min

Further investigations were undertaken to evaluate the function of the present systems. We combined **Camp-PC-biotin** and **SA–DNA complex** to form the **SA–Camp-PC-biotin–DNA complex**. We confirmed the formation of **SA–Camp-PC-biotin–DNA complex** using native PAGE (Figure 5). We observed the fluorescence from DNA aptamer and **Camp-PC-biotin** from the same position. In addition, the fluorescence from **Camp-PC-biotin** in the **SA–Camp-PC-biotin–DNA complex** disappeared after photoirradiation. Thus, the **SA–Camp-PC-biotin–DNA complex** has possibility to show cell toxicity under photoirradiation condition. Next, its cytotoxic effects upon

photoirradiation were evaluated (Figure 6c, d). SK-BR-3 cells were treated with **SA–DNA complex** with or without **Camp-PC-biotin**, and then the cells were subjected to photoirradiation. After cell culture for an additional three days, cell viability was measured; the results of the WST assay are shown in Figure 6d. Almost no cytotoxicity was observed for the **SA–DNA complex** alone, under either photoirradiation or non-photoirradiation conditions, because no drug unit was present. It was striking that stronger cytotoxic effects were observed in cells incubated with **SA–Camp-PC-biotin–DNA complex** under photoirradiation conditions than in cells without photoirradiation. These results strongly indicate that the drug release and associated cytotoxicity occurred even on the **SA–DNA complex** under photoirradiation conditions.

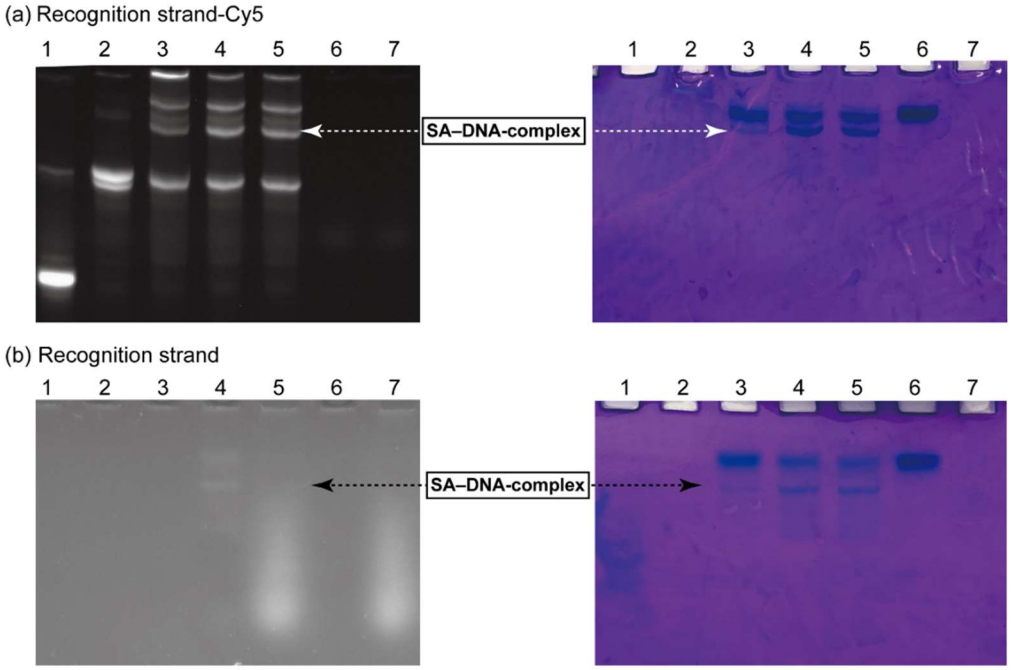


Figure 5 Evaluation of **SA–Camp-PC-biotin–DNA complex**. 8% native-PAGE analysis (a) Left: Cy5 monitoring (Ex. 530 nm). Right: CBB staining (Use of recognition strand-Cy5). (b) Left: Camptothecin monitoring (Ex. 375 nm). Right: CBB staining (Use of recognition strand without Cy5) Lane 1. 100 pmol recognition strand, Lane 2. **DNA complex** (100 pmol recognition strand + 100 pmol biotin strand + 100 pmol template strand), Lane 3. **SA–DNA complex** (100 pmol **DNA complex** + 100 pmol streptavidin), Lane 4. **SA–Camp-PC-biotin–DNA complex** (100 pmol **SA–DNA complex** + 100 pmol **Camp-PC-biotin**), Lane 5. **SA–Camp-PC-biotin–DNA complex** irradiated at UV light (365 nm) for 15 min. Lane 6. 100 pmol streptavidin, Lane 7. 100 pmol **Camp-PC-biotin** irradiated at UV light (365 nm) for 15 min.

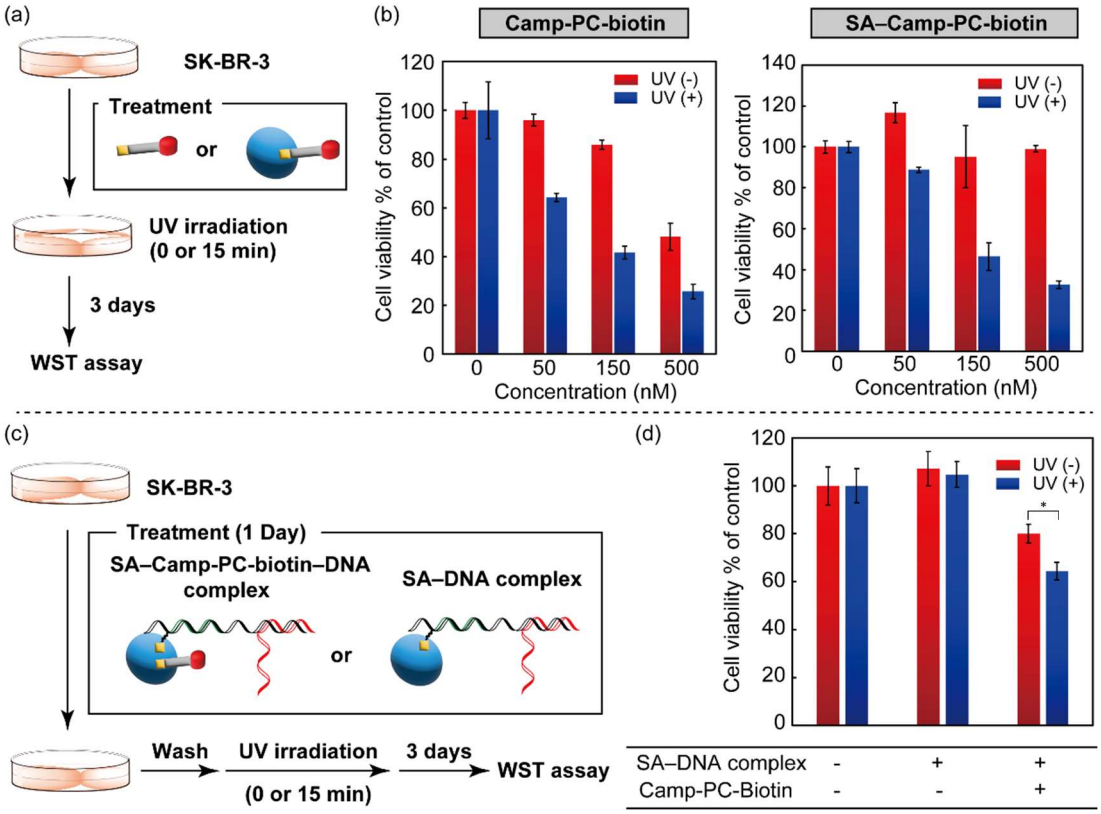


Figure 6. (a) Procedure of cell toxicity assays using the **Camp-PC-biotin** or **SA–Camp-PC-biotin**. (b) Cell viability of SK-BR-3 with or without photo irradiation (**Camp-PC-biotin** or **SA–Camp-PC-biotin**). Data = mean ± SE, n = 5. (c) Procedure of cell toxicity assay using the **SA–DNA complex** with or without **Camp-PC-Biotin**. (d) Cell viability of SK-BR-3 with or without UV irradiation (0, 500 nM **SA–DNA complex** or **SA–Camp-PC-biotin–DNA complex**). Data = mean ± SE, n = 5. (* indicates $p < 0.1$)

5-3 Conclusion

In conclusion, we designed, synthesized, and characterized a multifunctional prodrug of camptothecin. The prodrug was prepared with a DNA aptamer and camptothecin-based photo-activatable prodrug using a streptavidin scaffold. The prodrugs recognized membrane antigen and were smoothly taken into the cells. Photoirradiation resulted in

the release of drugs and consequent strong cytotoxicity.

DNA aptamers can be easily obtained by using the SELEX method for a variety of target antigens on the cell surface and in the cell itself. Therefore, the present system can be used in the design of several types of target-specific prodrugs by application of a suitable DNA aptamer. In addition, various photocleavable substituents can be included to construct the prodrug system. We are conducting research to improve and optimize the system to form prodrug constructs with high performance.

5-4 Experiment section

General Methods

Reagents were purchased from Wako pure chemical industries, Tokyo chemical industries, Sigma Aldrich, Nacalai Tesque, Dojindo and eurofins Genomics. The course of reaction was monitored by Thin-layer chromatography on silica gel plate (Silica Gel 60 F245). Wako Gel 60 was used for silica gel column chromatography. NMR spectra were recorded on a JNM-ECX500 Ⅱ (^{1}H: 500 MHz, ^{13}C:125 MHz) spectrometer (JEOL) and chemical shift are expressed in ppm downfield from tetramethylsilane using residual protons in the solvent as internal standards (Dimethyl sulfoxide-d_6: δ 2.49 in ^{1}H NMR, δ 39.5 in ^{13}C NMR). FAB mass spectrometry was performed with a JMS-700N mass spectrometer (JEOL), using nitrobenzyl alcohol as a matrix. Analytical High-performance liquid chromatographies were carried out with a D2000 HPLC system (HITACHI) and L7000 HPLC system (HITACHI) with reversed phase column. The column eluents were monitored by the absorbance at 365 nm at a flow rate of 0.6 mLmin^{-}

[1] with a 10–60% (over 60 min), 60–100% (over another 30 min) gradient of ACN/TEAA buffer (100 mM, pH 7.0). UV light irradiation was performed by High Performance Ultraviolet Transilluminator (funakoshi). Gel electrophoresis assay was captured by FluoroPhorestar 3000 (anatech). Absorption spectra were obtained using a V-650 UV-VIS Spectrophotometer (JASCO). A confocal microscopy (Nikon, confocal laser microscope system C2) was used for in vitro studies. Cell viability was recorded on a xMark^{TM} microplate absorbance spectrophotometer (BIO RAD). SK-BR-3 was purchased from American Type Culture Collection (ATCC). DNA sequences are shown in Table 1.

Table 1. DNA sequences used in this study

Names	Sequences (5' → 3')
Template strand	AGGTATGTATGCTTAGGGTCTTTTTTTTTTTTTTTTTTTT GAATTCCTGTAGATTGGACC
Biotin strand	GACCCTAAGCATACATACCT-Biotin
Recognition strand	GGTCCAATCTACAGGAATTCTTTTTTTTTTTTTTT CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTG
Biotin strand-FAM	FAM-GACCCTAAGCATACATACCT-Biotin
Recognition strand-Cy5	Cy5-GGTCCAATCTACAGGAATTCTTTTTTTTTTTTTTT CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTG
Recognition strand(Apt(-))-Cy5	Cy5-GGTCCAATCTACAGGAATTC

Synthesis

(S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 5-(((1-(4-(4-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-4-oxobutoxy)-5-methoxy-2-nitrophenyl)ethoxy)carbonyl)amino)pentanoate (Camp-PC linker, 2)

A solution of compound **1**[1] (494.3 mg, 903 μmol) in DCM (2.5 mL) was added of TFA (1.2 mL) at 0°C. The reaction mixture was stirred at room temperature for 1.0 h at ambient temperature. After reaction, the reaction mixture was evaporated. The crude product was dissolved in dry DMF (1.0 mL) and added of PC linker[2] (250.0 mg, 444 μmol) and Et_3N

(65 μL, 469 μmol) followed by stirring at room temperature for 2.0 h. The reaction mixture was extracted with $CHCl_3$. Then, organic layer was washed with brine and dried over $MgSO_4$. The crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH = 30/1) to give compound **2** (Camp-PC linker) (69.4 mg, 17%) as pale yellow solid. mp. 179.5-181.2°C; ^{1}H NMR (500 MHz, DMSO) : δ = 8.67 (s, 1H), 8.23 (s, 1H), 8.15 (d, 1H, *J* = 8.5 Hz), 8.10 (d, 1H, *J* = 9.0 Hz), 7.84 (m, 1H), 7.71 (m, 2H), 7.54 (d, 1H, *J* = 2.5 Hz), 7.11 (d, 1H, *J* = 3.0 Hz), 7.04 (s, 1H), 6.90 (s, 2H), 6.13 (m, 1H), 5.47 (s, 2H), 5.31 (s, 2H), 4.04 (t, 2H, *J* = 6.5 Hz), 3.87 (d, 3H, *J* = 4.5 Hz), 3.49 (t, 2H, *J* = 5.8 Hz), 3.23 (m, 2H), 2.52 (m, 2H), 2.48 (m, 2H), 2.18 (m, 4H), 1.93 (m, 2H), 1.60 (m, 2H), 1.49 (m, 5H), 0.94 (t, 3H, *J* = 7.5 Hz); ^{13}C NMR (125 MHz, DMSO) : δ = 172.5, 172.2, 171.6, 167.8, 157.0, 155.7, 154.0, 152.9, 148.4, 147.3, 146.5, 145.9, 139.7, 135.0, 134.0, 132.1, 131.0, 130.9, 130.4, 129.5, 129.0, 128.5, 128.2, 119.4, 109.0, 108.9, 95.2, 76.2, 68.8, 67.6, 66.8, 57.6, 50.8, 37.8, 37.4, 33.2, 32.1, 30.8, 29.0, 25.0, 22.4, 22.1, 8.1; FABMS (NBA) m/z : 895 $[M+H]^+$; HRMS calcd. for $C_{45}H_{46}N_6O_{14}[M+H]^+$ 895.3145, found 895.3202.

(S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 5-(((1-(4-(4-((2-(2,5-dioxo-3-((2-(5-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethyl)amino)pyrrolidin-1-yl)ethyl)amino)-4-oxobutoxy)-5-methoxy-2-nitrophenyl)ethoxy)carbonyl)amino)pentanoate (Camp-PC-Biotin, 4)

A solution of compound **3**[3] (63.2 mg, 164 μmol) in DCM (1.5 mL) was added of TFA (800 μL) at 0°C. The reaction mixture was stirred for 1.5 h at ambient temperature. After reaction, the reaction mixture was evaporated. The crude product was dissolved in dry

DMF (700 μL) and added of compound **2** (Camp-PC linker) (56.2 mg, 62.8 μmol) and Et_3N (90 μL, 649 μmol) followed by stirring for 3.5 h at ambient temperature. The reaction mixture was extracted with $CHCl_3$ and 1 N HCl aq. Then, organic layer was washed with brine and dried over $MgSO_4$. The crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH = 30/1) to give compound **4** (**Camp-PC-Biotin**) (40.1 mg, 54%) as pale yellow solid. mp. 153.6-154.5°C; ^{1}H NMR (500 MHz, DMSO) : δ = 8.66 (s, 1H), 8.23 (s, 1H), 8.14 (d, 1H, *J* = 9.0 Hz), 8.10 (d, 1H, *J* = 8.5 Hz), 7.84 (m, 1H), 7.70 (m, 2H), 7.55 (s, 1H), 7.50 (m, 1H), 7.18 (d, 1H, *J* = 3.5 Hz), 7.04 (s, 1H), 6.12 (m, 3H), 5.47 (s, 2H), 5.31 (s, 2H), 4.32 (t, 1H, *J* = 6.3 Hz), 4.15 (m, 1H), 4.05 (t, 2H, *J* = 6.3 Hz), 3.88 (d, 3H, *J* = 4.5 Hz), 3.77 (br, 1H), 3.54 (s, 1H), 3.46 (m, 2H), 3.24 (m, 2H), 3.17 (m, 2H), 3.09 (m, 4H), 2.86 (m, 2H), 2.71 (m, 1H), , 2.42 (m, 1H), 2.18 (m, 4H), 2.09 (t, 2H, *J* = 7.3 Hz), 1.95 (m, 2H), 1.59 (m, 9 H), 1.35 (m, 6H), 0.94 (t, 3H, *J* = 7.3 Hz); ^{13}C NMR (125 MHz, DMSO) : δ = 172.7, 172.5, 172.4, 167.8, 163.2, 157.1, 155.7, 154.0, 152.8, 148.5, 147.3, 146.5, 145.9, 139.7, 133.9, 133.9, 132.1, 130.9, 130.3, 129.5, 129.0, 128.5, 128.2, 119.4, 109.0, 108.8, 95.3, 79.7, 76.2, 70.3, 68.8, 67.6, 66.8, 61.5, 59.7, 56.6, 55.9, 50.8, 46.7, 46.1, 38.4, 36.6, 35.7, 33.2, 32.1, 31.5, 30.8, 29.0, 28.7, 28.5, 25.7, 25.0, 22.6, 22.4, 22.1, 14.5, 8.1; FABMS (NBA) m/z : 1182 $[M+H]^+$; HRMS calcd. for $C_{57}H_{68}N_{10}O_{16}S$ $[M+H]^+$ 1181.4635, found 1181.4635.

Monitoring of photo cleavage reaction

Monitoring of camptothecin release using Camp-PC-Biotin upon photo irradiation

A solution of 10 μM **Camp-PC-Biotin** in 10 mM pH7.4 Phosphate buffer containing 10% DMSO was irradiated at UV light (365 nm) for 0 or 15 min. The sample was

incubated for 0–72 h at 37°C in the cell incubator (5% CO_2 / 95% air) and analyzed by HPLC.

Cellular experiment

Cell culture

SK-BR-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were maintained at 37 °C in 5% CO_2 and were kept in the logarithmic growth phase by routine passages every 3–4 days. Prior to the use of cells, the densities of cells were determined using a hemocytometer.

Formation of SA–DNA complex and complex with Camp-PC-Biotin

To form **DNA complex**, 10 or 50 μM template strand, biotin strand and recognition strand in D-PBS containing 5 mM $MgCl_2$. were mixed and heated to 90 °C for 5 min, and then, gradually cooled to ambient temperature.

Figure 3

The 1 μM **DNA complex** and 1 μM streptavidin was mixed in D-PBS containing 5 mM $MgCl_2$ and incubated at ambient temperature for 30 min. Formation of **DNA complex** and **SA–DNA complex** was evaluated by agarose gel electrophoresis (3.5 % agarose containing GelRed).

Figure 5

The 10 μM **DNA complex**, streptavidin and Camp-PC-biotin was mixed in D-PBS containing 5 mM $MgCl_2$ and incubated for 1 h at ambient temperature. Formation of **SA–Camp-PC-biotin-DNA complex** was evaluated by 8% native PAGE.

Figure 6c, d

Complex with **Camp-PC-Biotin** (Figure 4c, d) was prepared by mixing the 500 nM **DNA complex**, streptavidin, and **Camp-PC-Biotin** in DMEM and incubated for 1 h at ambient temperature.

Fluorescence observation using living cell

DNA complex, **SA–DNA complex** and **SA–DNA complex (Apt (-))** were prepared as described above. SK-BR-3 cells (1.5×10^4 cells per well) were seeded onto glass bottom dish in DMEM. The cells were cultured at 37 °C in 5% CO_2 / 95% air for 24 h. Cells were washed with D-PBS containing 5 mM $MgCl_2$, and then, the samples (200 nM, 60 μL) were added into each well and incubated for 10 min at 4°C. The cells were washed with D-PBS containing 5 mM $MgCl_2$, and then observed by confocal microscopy. (FAM: Excitation = 488 nm, Cy5: Excitation = 640 nm).

Cytotoxicity evaluation using Camp-PC-Biotin upon photo irradiation

SK-BR-3 cells (5.0×10^3 cells per well) were seeded onto 96 well plate in DMEM. The cells were cultured at 37 °C in 5% CO_2 / 95% air for 24 h. The indicated concentration of **Camp-PC-Biotin** or **SA–Camp-PC-Biotin** in DMEM containing 1% DMSO (50 μL) were added into each well and irradiated at UV light (365 nm) for 0 or 15 min at ambient temperature. After photo irradiation, 50 μL of fresh medium was added into each well and incubated at 37 °C in 5% CO_2 / 95% air for 72 h. After incubation, 10 μL of Cell Counting Kit-8 solution (Dojindo) were added to each well and the cells were cultured at 37°C in 5% CO_2 / 95% air for 40 min. Absorbance at 450 nm was recorded on a microplate absorbance spectrophotometer.

Cytotoxicity evaluation using complex with prodrug

SK-BR-3 cells (7.5×10^3 cells per well) were seeded onto 96 well plate in DMEM. The cells were cultured at 37 °C in 5% CO_2 / 95% air for 24 h. 50 μL of 500 nM **SA–DNA complex** or complex with **Camp-PC-Biotin** were added into each well and incubated for 24 h at 37 °C in 5% CO_2 / 95% air. After washing with D-PBS(-) twice, cells were irradiated at UV light (365 nm) for 0 or 15 min at ambient temperature. After treated with UV light, 50 μL of fresh medium was added into each well and incubated at 37 °C in 5% CO_2 / 95% air for 72 h. After incubation, 10 μL of Cell Counting Kit-8 solution (Dojindo) were added to each well and the cells were cultured at 37°C in 5% CO_2 / 95% air for 30 min. Absorbance at 450 nm was recorded on a microplate absorbance spectrophotometer.

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Chapter 1

Encoding thiol metabolite information into a DNA sequence by disulfide exchange reaction on oligodeoxynucleotides for parallel analysis of the metabolite and mRNA.

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Chapter 3

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Chapter 5

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Other Publication

(1) pH-responsive aggregate consisted of oligodeoxynucleotides bearing nitrophenol group that deliver drugs into acidic cells. S. Yokota, Y. Motohashi, T. Nishihara, K. Tanabe, *Bioorg. Med. Chem. Lett.* **2022**, 58, 128519.

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(2) ○ Yuto Motohashi, Shuhei Moritani, Tatsuya Nishihara, Kazuhito Tanabe, “Development of DNA–protein complex aiming high selective recognition of membrane antigens” The 100th CSJ Annual Meeting, Noda campus, Tokyo university of science (2020. 3. 22.)

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