研究説明資料

論文題目

Construction of biomacromolecular assemblies with spatially arranged functional units to assess the cellular functions

(機能性ユニットを空間的に配置した生体高分子組織体による細胞内機能プローブの構築)

京都大学大学院エネルギー科学研究科

エネルギー基礎科学専攻

Zhang Zhengxiao

In this thesis, several conjugation strategies, including chemoselectivity and recognition-driven conjugation, were utilized to modify functional units of interest on biomacromolecules to construct biomacromolecular assemblies for evaluating cellular processes. The recognition-driven conjugation directs site-specific conjugation of over three modular adaptors on a DNA scaffold for realizing artificial enzyme cascades. Fluorescent sensors for the simultaneous detection of multiple types of cathepsins and pH sensors were developed using the biomacromolecular assemblies, DNA nanostructures, or dextran by realizing the spatial arrangement of functional units on the assemblies through the chemoselective conjugation reactions.

Contents of this thesis

Chapter 1 is a general introduction that describes the background of research carried out in this thesis. Through conjugation reaction, biomacromolecules can be functionalized as biomacromolecular assemblies for diverse cellular studies. DNA

scaffolds and polysaccharides emerged as ideal biomacromolecular scaffolds for functionalization through different conjugation strategies. For this purpose, two strategies of conjugation are intensively involved in modifying the DNA scaffolds and polysaccharides: chemoselective conjugation and recognition-driven conjugation. The former is exploited to realize selectivity by modifying the nucleophilic group or chemical handle on DNA scaffolds or polysaccharides. In contrast, the recognition-driven conjugation relies on the recognition nature of proteins to achieve the sequence-specific conjugation of proteins on the DNA scaffolds.

In Chapter 2, I developed a set of modular adaptors (MA) and their substrates based on SNAP-tag to realize recognition-driven DNA-protein conjugation. The protein tags, such as SNAP-tag and CLIP-tag, were combined with DNA binding domain (DBD) to construct MA, which couples the molecular recognition event (via DBD) to the chemoselective reaction between the protein tag and substrate on DNA to realize a far greater reactivity and selectivity. Owing to the extremely high reaction rate between SNAP-tag and its original substrate benzylguanine (BG), the reaction between modular adaptors consisting of DBD and SNAP (MA-SNAP) and ODN modified by BG failed to proceed in the sequence-selective manner. In order to reduce the reactivity of SNAP-tag to its substrate, two BG derivatives, i.e., benzylinosine (BI) and 7-deaza-benzylguanine (deBG), were designed by altering the interaction sites of BG with SNAP-tag. Both substrates exhibited the reduced rate constants for the reaction with SNAP-tag. For further characterization of the sequence-selective reaction of MA, BI was incorporated into DNA to elucidate the kinetic details of sequence-selective modification by MA-SNAP. As a result, I demonstrated sequence-selectivity of MA-SNAP with BI-modified DNA from three aspects: sequence-selective conjugation among multiple DNA sequences; estimation of all kinetic parameters to conclude $k_{cov} \ll k_{off}$; proportional relationship

between k_{app} and K_D. Those results strongly proved that k_{cov} of MA reaction is a vital key for achieving the recognition-driven conjugation between MA and DNA.

In Chapter 3, a strategy that can spatially arrange over four unique MAs on a DNA scaffold was verified. Considering that modular adaptors consisting of CLIP-tag (MA-CLIP) provided up to three orthogonal sequence-selective MAs in combination with three distinct sequence-selective DBD (two types of zinc finger domains and a basic leucine-zipper domain), the newly designed three types of MA-SNAPs with the use of BI exploited another three types of sequence-selective modular adaptors. Fortunately, high discrimination of BI against CLIP-tag prevented the BI-modified ODN from nonspecific reaction with MA-CLIP. By three types of DNA binding sequence modified with BI or benzylcytosine (BC), I was able to specifically and efficiently react the target substrate-modified DNA with only one type of MA among six unique MAs, namely, only the reaction of MA with matched DBD, and the substrate proceeded. Therefore, the chemoselectivity between SNAP- and CLIP-tags enabled by the application of BI allowed us to use each three MA-SNAPs and MA-CLIPs simultaneously, which doubled the number of orthogonal modular adaptors. With the efficient and specific DNA-protein conjugation mediated by MA, both sequence-selective conjugation and chemoselective conjugation of MA could be used to locate proteins of interest on the same DNA scaffold. A breakthrough result was accomplished experimentally when two orthogonal series of MA were applied, where four different MAs were orthogonally located at the respective target sites on a DNA scaffold. This result provided the technical basis for constructing enzyme cascades consisting of at least four different enzymes on a DNA scaffold.

In Chapter 4, to detect cathepsin B (CtB) and D (CtD), orthogonal FRET-based probes were conjugated with a DNA handle for their modification on a biomacromolecular scaffold, such as the DNA scaffold. CtB, a major lysosomal cysteine

protease, is an essential biomarker for aggressive cancers. CtD, an aspartic protease inside the lysosome, is processed to mature cells by subsequent proteolytic cleavage involving CtB and other cysteine proteases. Coupling the CtB sensor with the CtD sensor enables clarification of the correlation between the activity of CtB and CtD. To construct sensors for cathepsin B and D, peptidic substrates for CtB and CtD were synthesized and modified with orthogonal FRET pairs for directly sensing the activities of CtB and CtD. These two cathepsin probes showed sufficient reactivity and specificity to detect the respective target enzymes. As a DNA handle was conjugated with orthogonal FRET-based CtB probe and CtD probe, the conjugated DNA handle provided programmability to cathepsin probes, enabling the probes to be co-assembled on DNA scaffolds. Interestingly, the DNA handle conjugated with FRET-based cathepsin probes showed drastic improvement of the reactivity toward cathepsins over their parent probes. It is likely that the conjugated DNA handle improved the solubility of the probes with hydrophobic and bulky fluorescent groups. The fact manifested that introducing the DNA handles to FRET-based probes not only expanded its application to assemble on the scaffold, but also mitigated the disadvantage owing to the hydrophobicity of FRET pairs, enabling various types of FRET pairs used in the design of the protease probes. To use the cathepsin sensor with a pH sensor simultaneously, I modified SNARF fluorophores with maleimido, which enabled the SNARF fluorophore to be modified to a DNA handle. The resulting SNARF fluorophore modified with a DNA handle was further assembled on a DNA scaffold as a ratiometric fluorescent pH probe. The pH sensor on the DNA scaffold was applied for the real-time sensing of cellular pH changes.

In Chapter 5, I presented a dual-reporter approach to the cellular imaging of activated CtB in the lysosome. CtB is known to be overexpressed in various cancers, particularly in aggressive cancers, making it an attractive target for tumor-specific

prodrug design. In the acidic environment of lysosomes, procathepsin B undergoes autocatalytic activation, leading to the formation of active CtB. Alternatively, activation of CtB can be correlated with active CtD, which is an aspartic endo-protease universally found in lysosomes. Therefore, a reporter of the acidic environment or that of CtD would provide useful information for characterizing the activated CtB. The peptidic substrates for CtB and CtD were synthesized and modified with FRET pairs for directly sensing the activity of CtB and CtD as described in Chapter 4. A ratiometric fluorescent pH probe was prepared by assembling two types of fluorophores on dextran to report the pH around the cellular environment to detect the protease activity. Upon characterizing each sensor, conjugation of the cathepsin sensor on dextran dramatically improved its solubility and availability for sensing the activity of target cathepsin. Coupled with a pH sensor on dextran, the probe was able to detect the activity of cathepsin under different pH for both *in vitro* and *in vivo* applications.

In Chapter 6, I summarized the overall conclusions based on the results described in Chapters 2 to 5. For introducing protein on the biomolecular scaffold, the recognition-driven reaction of modular adaptors was studied from the kinetic aspects. It was established that two orthogonal series of modular adaptors consisting of SNAP-tag or CLIP-tag provided at least four unique modular adaptors to orthogonally target the specific sites on a DNA scaffold. FRET-based orthogonal cathepsin probes were designed and conjugated with the DNA handle, which not only promoted the capability of orthogonally sensing cathepsin B and D, but also facilitated their assembly on the DNA scaffold. The FRET-based cathepsin probes were coupled with pH sensors by conjugating on dextran to detect lysosomal active cathepsin B activated in an acidic condition *in vitro* and *in vivo*. The results presented in this thesis demonstrated that chemoselective and

recognition-driven conjugations are significantly useful tools to functionalize biomacromolecular scaffolds with functional units for studying the cellular processes.

Significance of research

In this research, I took advantage of chemoselective reaction and recognition-driven DNA-protein conjugation to (1) locate more than four types of proteins on a DNA scaffold, (2) assemble FRET-based cathepsin sensors on a DNA scaffold, and (3) functionalize polysaccharides with pH sensors and cathepsin sensors. This study demonstrated the crucial roles of diverse conjugation reactions for precisely functionalizing biomacromolecular scaffolds with various functional units, such as fluorophores, peptides, and proteins. Especially, the spatial control of over four types of proteins on a DNA scaffold provides a new strategy for chemically investigating *in vitro* complex enzyme cascades, which provide the basis of artificial metabolic processes in the carbon-neutral society. At the same time, biomacromolecular scaffolds feature good biocompatibility and multiple functions are essential platforms for disease diagnosis and drug delivery. The rational application of conjugation reactions for functionalizing biomacromolecular scaffolds would promote further development of pharmaceutical and clinical research.

Future perspectives

Conjugation reactions for constructing functional biomacromolecular assemblies will boost our studies of various cellular processes for both *in vitro* and *in vivo* investigations. For *in vitro* studies, complex enzyme cascades, including over four different types of enzymes, could be spatially arranged on a DNA scaffold using appropriate modular adaptors. With those assemblies, complex enzyme cascades are elucidated kinetically and quantitatively to set a basis for new material production

processes suitable for the carbon-neutral society. For *in vivo* studies, dextrans showed excellent characteristics as the vehicle to lysosome. Various lysosomal biomarkers will be targeted by modifying dextran with appropriate sensors for prospective applications in the early detection of tumors. With further functionalization of the biomacromolecular scaffolds, the biocompatibility and multiple functionalities of biomacromolecular assemblies would provide new tools for disease diagnosis and therapy.

List of publications

Publications directly related to the contents of this thesis

1. RNA-peptide conjugation through an efficient covalent bond formation

Shun Nakano, Taiki Seko, Zhengxiao Zhang and Takashi Morii

Appl. Sci., **2020**, *10*, 8920-8929.

(Chapter 1)

2. Tuning the reactivity of substrate for SNAP-tag expands its application for recognition-driven DNA-protein conjugation.

Zhengxiao Zhang, Eiji Nakata, Huyen Dinh, Masayuki Saimura, Arivazhagan Rajendran, Kazunari Matsuda and Takashi Morii

Chem. - Eur. J., **2021***,27,* 18118-18128.

(Chapter 2, 3)

3. FRET-Based cathepsin probes for simultaneous detection of cathepsin B and D Activities

Zhengxiao Zhang, Eiji Nakata, Yuya Shibano and Takashi Morii

ChemBioChem., **2022**, *In press*. https://doi.org/10.1002/cbic.202200319

(Chapter 4)

4. Orthogonal FRET reporters for the real-time sensing of lysosomal proteases

Zhengxiao Zhang, Eiji Nakata, Yuya Shibano, Hisaaki Hirose, Shiroh Futaki, Takashi Morii

Manuscript in preparation

(Chapter 5)

Publications not directly related to the contents of this thesis

1. Rational design of DNA sequence-specific modular protein tag by tuning the

alkylation kinetics

Thang Minh Nguyen, Eiji Nakata, Zhengxiao Zhang, Masayuki Saimura, Huyen Dinh and Takashi Morii

Chem. Sci., **2019***, 10,* 9315-9325.

2. Evaluation of the role of the DNA surface for enhancing the activity of scaffolded enzymes.

Peng Lin, Huyen Dinh, Yuuki Morita, Zhengxiao Zhang, Eiji Nakata, Masahiro Kinoshita and Takashi Morii

Chem. Commun., **2021***, 57,* 3925-3928.

3. A facile combinatorial approach to construct a ratiometric fluorescent sensor: application for the real-time sensing of cellular pH changes

Eiji Nakata, Hisaaki Hirose, Khongorzul Gerelbaatar, Jan Vincent V. Arafiles, Zhengxiao Zhang, Shiroh Futaki and Takashi Morii

Chem. Sci., **2021***, 57,* 8231-8240.