早稲田大学大学院 先進理工学研究科

博士論文審査報告書

論　文　題　目

Modifying DNA Origamis for Increased Size and Disease Detection Functionality

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1. Outline of Doctoral Thesis

DNA origami has been considered as a potentially important tool in nanotechnology for efficient delivery of drugs into a biological system. Nevertheless, prior to its application, DNA origami still faces outstanding challenges particularly in scalability and functionality. This doctoral thesis reports an attempt to address these challenges by establishing a novel DNA origami motif assembled from catenated ssDNA, called DNA topogami (scalability) and by producing a DNA origami scaffold able to selectively bind to *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH), an important diagnostic indicator for all malaria-causing *Plasmodium* parasites. This opens the possibility for building more advanced and complex devices, with potential use as diagnostic tools and which may also provide the basis for drug delivery systems for malaria (functionality).

In order to develop the novel DNA origami motif termed "DNA topogami", topologically interlocked catemers were constructed using an M13mp18 DNA scaffold in a preliminary catenation step mediated by human ENA/VASP-like (EVL) protein and catalyzed by *Escherichia coli* topoisomerase I. Subsequent folding of the fabricated DNA catenanes with staple strands would enable the assembly of mechanically interlinked topological structures, larger in size than regular DNA origami tiles. In comparison to the existing scaling up approaches, DNA topogami promises to generate mechanically interconnected, robust structures assembled via a single set of staple strands, capable of folding each and every scaffold *catena* simultaneously, in a single one-pot reaction, therefore greatly reducing the cost of production. Additional advantages of this method include the possibility to finely tune the size of the assembly through adjusting the size of the scaffold during catenation. This is a significant advance considering that most existing scaling methods rely on using numerous sets of staples for the step-wise assembly of each integrated component. Moreover, step-wise techniques generate intrinsically weak superstructures, due to the fault line between the assembled structures and the weak nature of bonding between tiles. Structurally similar to the DNA origami concept, DNA topogami hold the advantage of coordinated scalability through exercising control over the degree of preliminary scaffold catenation.

In order to address the functionality challenge, a DNA aptamer was incorporated into a DNA origami tile for the selective capturing and immobilization of *Pf*LDH, an important diagnostic indicator for all malaria-causing *Plasmodium* parasites. While the incorporation of various proteins onto the DNA origami tile has been previously described, this was the first attempt to use *Pf*LDH in conjunction with the aptamer modified DNA origami (AM-DNA origami) system, with the objective to engineer a device capable of detection and potential future drug delivery. The structural design was based on a standard DNA origami rectangle and employed aptamer sequences known to bind to *Pf*LDH with high specificity and avidity. In order to incorporate these aptamers into DNA origami, a staple sequence complementary to part of the M13 DNA origami template strand was added at the 5′end of the aptamer. 12 modified aptamers were incorporated into the rectangle through 12 staple sequences complementary to M13 DNA at positions that formed a linear pattern along one edge of the DNA rectangle. Atomic force microscopy demonstrated that the incorporated aptamers preserved their ability to specifically bind target protein. Captured *Pf*LDH retained enzymatic activity and protein-aptamer binding was observed dynamically using high-speed AFM. This work demonstrates the ability of DNA aptamers to recognize a malaria biomarker whilst being integrated within a supramolecular DNA scaffold, opening new possibilities for malaria diagnostic approaches based on DNA nanotechnology. Additionally, this system could be further developed into a smart drug delivery nanodevice containing a drug molecule that would be released for malaria treatment. The method of incorporation would depend on the nature of the drug and target molecules, being released only in the presence of disease related parasites.

2. Contents of the Doctoral Thesis Public Defense

On the 13th of May 2016, a public defense was held as a follow-up to the preliminary defense, in order to validate the contents of the submitted doctoral thesis. The discussions, questions and answers presented during the public defense are listed below:

1. A question was asked regarding the two-step assembly process where 12 staple strands were omitted from the DNA origami structure, and why the removal of those 12 staples did not affect the overall structural integrity of the origami tile. The candidate presented the following answer: DNA origami was folded with the aid of over 200 staple strands. Removing 12 oligonucleotides would not affect the overall structural integrity of the DNA origami tile. Although the intermediate partially folded DNA origami was not visualized via AFM, if imaged, a fully assembled DNA origami tile with a partially folded edge would be observed, due to the absence of 12 staples. The unfolded M13 strand would fully integrate into the origami tile structure upon addition of the 12 remaining staples.
2. A question was made on how DNA origami could be used as a potential tool for malaria treatment in addition to its application in diagnostics. To answer this question, the candidate presented an example of a previously constructed DNA origami structure shaped as a hexagonal barrel. It was constructed from two domains linked covalently at the rear edge by DNA scaffold hinges, and closed at the front via an aptamer-based lock mechanism. Upon reacting with a set of antigen keys, the aptamers would act as entropic springs and reconfigure the barrel to give access to its interior surface, unloading the cargo. The barrel could be loaded in order to contain either a signaling or a drug molecule, which would be released either for diagnosis or treatment.
3. The comments were made that if such DNA barrels were to be used, only several drug molecules would be delivered into the blood stream. It was asked whether that would be enough for treatment purposes. The candidate answered that this problem could be addressed by adjusting the concentration of the DNA origami solution. An alternative strategy would be to increase the size of the DNA origami barrel in order to accommodate a higher concentration of drug molecules. In general terms, the entire design of the DNA origami structure would have to be adjusted so as to fit the size of the drug molecules. Furthermore, the biodegradability of DNA origami in the blood stream was also discussed.
4. Regarding further applications of this system, a question was asked about detecting the protein-aptamer binding reaction in areas with limited resources without access to AFM. The candidate answered that an easily observable signal would have to be developed, e.g. a colorimetric assay based on the intrinsic enzymatic activity of *Pf*LDH. The results presented in this thesis show that *Pf*LDH bound to AM-DNA origami retained enzymatic activity and was stable in the presence of human plasma. This will facilitate the future integration of the AM-origami protein system into nanometric devices capable of detection based on localized enzymatic activity. A more complex origami device may be able to encapsulate and separate molecules that produce a functional fluorophore only when they interact. In such a system, aptamers could actuate opening of the encapsulating DNA origami, allowing the molecules to interact and produce a fluorescent signal.
5. A question was made on the merit of using DNA origami as a detection system. The candidate mentioned that as a diagnostic tool, DNA origami has advantages in simplicity of design and assembly, small size, addressability, programmability, and the ability to incorporate multiple copies of the same aptamer simultaneously, thereby enhancing detection sensitivity. Alternatively, it could incorporate several different aptamers for distinct ligands, thus increasing sophistication and subtlety of detection. The biggest limiting factor at the moment seems to be cost, however, this issue has not been considered from an engineering perspective. The volume necessary for detection lies in the microliter region, therefore, if production was to be scaled up and optimized, and the cost per reaction calculated, it could be that the expense is similar to the currently available detection options.

6. A question was asked about the binding on/off rates, specifically why the calculated Kds were high, yet HS-AFM results indicated towards strong binding affinity. The candidate answered that the calculated Kd corresponded to the aptamer modified staple strands prior to integration into the DNA origami tile, as the tile was too large to be run on the gel. Therefore, the exact binding Kd would differ based on the fact that non-interacting sequences at the termini of aptamers resulted in a decreased affinity for their targets, likely due to additional unwanted interactions, fact previously reported. Once those termini sequences were integrated into the DNA origami structure during assembly, the actual Kd would differ significantly from the calculated values, being expected to correspond to the original reported lower Kd values.
7. A question was asked whether any publications were available showing DNA origami keeping its structural integrity and not being degraded in the blood stream. The candidate explained that the stability of DNA origami has been tested in cell lysates from various normal and cancerous cell lines, and the published papers reported DNA origami to retain its structural integrity. Additionally, the DNA could be separated from cell lysates without damage or degradation.
8. A question was made whether HS-AFM required static or dynamic conditions. As the answer, the candidate mentioned that for imaging purposes the sample was added directly to the mica surface. However, the microscope contained a chamber to which buffer or proteins could be further added, therefore the system could be compared to a microfluidic device and the process considered dynamic, although sample could not be added to the mica surface itself without stopping the imaging process.

Based on the explanations to the presented questions the candidate has provided sufficient answers and sound discussion to further allow the examiners to understand and interpret her doctoral thesis research. We believe that she has provided critical and new insights to the field of DNA Nanotechnology and believe that her contributions will assist in the advancement of this field. Additionally, the candidate has included the answers to the questions presented during the defense into the doctoral thesis, in order to allow future readers to better comprehend her research. Therefore, after severely evaluating the candidate's doctoral thesis and her preliminary and public defense, we agree that the candidate has sufficient knowledge and should be awarded the Doctor of Science degree.

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Principal Referee	Haruko Takeyama, Professor,Waseda University Doctor of Engineering (Tokyo University of Agriculture and Technology)
Referee	Shinji Takeoka, Professor,Waseda University Doctor of Engineering (Waseda University)
Referee	Takafumi Inoue, Professor,Waseda University Doctor of Philosophy in Medical Science (Osaka University)
Referee	Jonathan Heddle, Professor, Jagiellonian University (Poland) PhD (University of Leicester, UK)