Programmable protein-protein conjugation via DNA-based self-assembly

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Programmable protein-protein conjugation via DNA-based self-assembly

Josui Shimada,[a] Tatsuo Maruyama,[b,*] Momoko Kitaoka,[a] Hisao Yoshinaga,[a] Koji Nakano,[a] Noriho Kamiya,[a,c] and Masahiro Goto[a,c,*]

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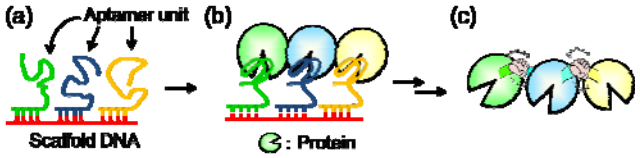
Protein molecules were precisely arrayed on a desinable DNA scaffold close to each other using a DNA aptamer. By adding a chemical cross-linker, the neighboring protein molecules were effectively and covalently cross-linked to each other without losing their activities.

In nature, proteins assemble and display biocatalytic cascades such as the sequential multistep transformation of a substrate. It is important that the component proteins are located in the proximity of the active site of each protein to effectively catalyze the overall biotransformation.[1] Studies of neighboring protein-protein communications and the development of artificial multi-enzyme complexes for potential applications in biotechnology, including bioproduction, biosensing, bioelectronics, and biofuel cells have provided significant research challenges.[2]

DNA has garnered attention as a promising engineering material for bottom-up nanofabrication in the fields of nanotechnology and nanodevices.[3] Highly-ordered DNA nanostructures can precisely arrange target molecules and substrates, such as low-molecular-weight drugs,[4] metal nanoparticles[5] and proteins,[6] with controlled intervals and at aimed positions. In particular, the use of DNA scaffolds for the assembly of proteins has attracted research interest.[7] To date, there are reports on the self-assembly of enzyme-oligonucleotide conjugates on DNA scaffolds,[8] which indicates that the appropriate spatial arrangements of proteins can intensify the inherent functional properties of proteins and create other functional properties. DNA molecules (termed DNA aptamers) can also recognize and capture non-DNA target molecules.[9] DNA scaffolds containing DNA aptamers have been used for the arrangement of proteins onto one-dimensional,[10] periodic protein-nucleic acid nanostructures[11] or the construction of protein-oligonucleotide nanowires.[12] However, the reported assemblies of proteins can be easily decomposed by degradation of the DNA scaffolds and it has been difficult to separate the proteins assemblies from the DNA scaffolds, and hence, this has limited the application of such assemblies.[13]

Here, we present the covalent protein-protein conjugation programmed by synthesized DNA as illustrated in Scheme 1. To demonstrate the concept, we chose thrombin as a model protein for the protein conjugation and designed four different



Scheme 1 Schematic representation of the DNA-templated protein-protein conjugation. (a) The aptamer strands hybridize with the DNA scaffold at their sticky ends. (b) Proteins are site-specifically associated with aptamer strands on the DNA scaffold. (c) The covalent protein-protein conjugation and DNA removal.

single-stranded DNA. One of them is a template of a DNA scaffold for the arrangement of the thrombin-binding aptamers[14] and three of them are thrombin-binding aptamers with different sticky ends that can hybridize with the DNA template (aptamer unit). By self-assembling these oligonucleotides, they should form a comb-like structure (termed DNA scaffold) composed of both the linear chains due to the DNA double-strands and the branched arms (the flexible DNA aptamer units) (Scheme 1a). Thrombin molecules can get close to each other by the binding of the thrombin molecules with the aptamer units (Scheme 1b). Then, the addition of a chemical cross-linker induces the neighboring thrombin molecules on the DNA scaffold to easily cross-link each other (Scheme 1c).

According to the crystalline structure of thrombin, the diameter of thrombin is around 4 nm and many positively-charged amino acid residues are located around exosites I and II (binding sites of the thrombin-binding aptamer).[15] Therefore, the DNA template and the aptamer units are designed as shown in Table S1. The aptamer units are composed of the sticky ends of 12 bases (~4.1 nm), the spacer sequence of 5 dTs and the 15-mer thrombin-binding aptamer.[14]

To investigate the aptamer-based assemblies of thrombin molecules on DNA scaffolds (Scheme 1b), the DNA scaffolds were mixed with thrombin molecules and analyzed by native polyacrylamide gel electrophoresis (Fig. 1). As the kinds of the aptamer units (A1, A2 and A3) increased, the new bands appeared in the lower electrophoretic mobility region. Lane 7 shows the same electrophoresis profile as in lane 1, indicating that the template DNA did not interact with thrombin. Lane 8

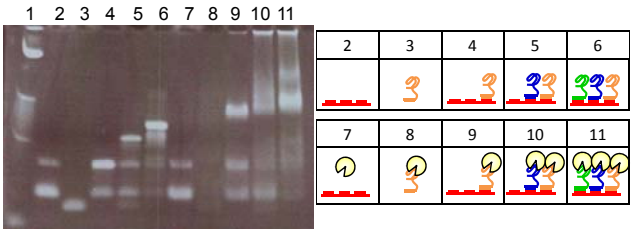


Fig. 1 Native polyacrylamide (10%) gel electrophoresis (PAGE) analysis of DNA scaffolds and DNA-thrombin complexes. DNA sequences used are summarized in Table S1. Lane 1: 10 bp DNA ladder; lane 2: template DNA-only; lane 3:A1-only (32-mer DNA). Mixture solutions of template DNA with A1 (lane 4); template DNA with A1 and A2 (lane 5); template DNA with A1, A2, and A3 (lane 6); template DNA with thrombin (lane 7); A1 with thrombin (lane 8); template DNA, and A1 with thrombin (lane 9); template DNA, A1, and A2 with thrombin (lane 10); template DNA, A1, and A2 and A3 with thrombin (lane 11).

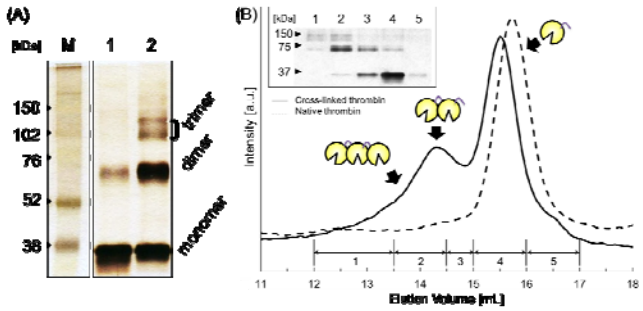


Fig. 2 Cross-linking of thrombin molecules using BS3 (50-fold molar excess to thrombin) and analysis by 7.5% non-reducing SDS-PAGE (A). (M) Protein rainbow marker, (1) cross-linking in the absence of the DNA scaffold, (2) cross-linking in the presence of the DNA scaffold. SEC charts (B) of the cross-linked thrombin (solid) and native thrombin (dashed line). Each fraction was analyzed by 10% non-reducing SDS-PAGE (inset). Fraction 1 is the elution volume of 12–13.5 ml, fraction 2 is that of 13.5–14 mL, fraction 3 is that of 14–14.5 ml, fraction 4 is that of 14.5–15 mL, and fraction 5 is that of 15–16 ml. Gel was stained using a silver stain kit.

showed that an original band of aptamer unit A1 disappeared. For this PAGE analysis, the DNA was stained by SYBR Green II. The formation of the aptamer unit/thrombin-complex might interfere with the intercalation of SYBR Green II to the DNA and could be one of the possible reasons for the absent band. Lanes 9–11 shows that the original bands were drastically shifted in the presence of thrombin molecules. These results support our hypothesis of the aptamer-based assembly illustrated in Scheme 1b.

To investigate the aptamer-based assemblies of thrombin molecules on DNA scaffolds (Scheme 1b), the DNA scaffolds were mixed with thrombin molecules and analyzed by native polyacrylamide gel electrophoresis (Fig. 1). As the kinds of the aptamer units (A1, A2 and A3) increased, the new bands appeared in the lower electrophoretic mobility region. Lane 7 shows the same electrophoresis profile as in lane 1, indicating that the template DNA did not interact with thrombin. Lane 8 showed that an original band of aptamer unit A1 disappeared. For this PAGE analysis, the DNA was stained by SYBR Green II. The formation of the aptamer unit/thrombin-complex might interfere with the intercalation of SYBR Green II to the DNA and could be one of the possible reasons for the absent band. Lanes 9–11 shows that the original bands were drastically shifted in the presence of thrombin molecules. These results support our hypothesis of the aptamer-based assembly illustrated in Scheme 1b.

Since the distance between neighboring thrombin molecules captured on the DNA scaffold was designed to be close, it would be easy to cross-link thrombin molecules only with neighboring thrombin molecules on the DNA scaffold using the chemical cross-linker, BS3. BS3 has a spacer arm of 1.14 nm in length, which can react with protein primary amino groups. BS3 was added to the DNA scaffold-thrombin complex solution and the reaction products were subjected to non-reducing SDS-PAGE, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and size-exclusion chromatography (SEC). Lane 1 of Fig. 2(A) shows that it was hard to cross-link thrombin molecules with each other in the absence of DNA scaffolds. Thrombin has a molecular weight of ~37 kDa and contains a carbohydrate portion with N-linked glycosylation.[16] The molecular weights of thrombin dimer and thrombin trimer should be ~74 kDa and ~111 kDa. Lane 2 of Fig. 2(A) shows that the dimer and trimer of thrombin were seemingly constructed in the presence of the DNA scaffold. However, there seem to be two bands observed for thrombin trimer. We examined the molecular weights of thrombin oligomers by MALDI-TOF-MS and SEC. The MALDI-TOF-MS spectrum (Fig. S1) shows that in the presence of the DNA scaffold, peaks of the cross-linked thrombin were observed around m/z 72,144 and m/z 107,962 (inset of Fig. S1), suggesting the presence of thrombin dimer and trimer.

After the cross-linkage of thrombin on the DNA scaffold using BS3, another strand complementary to the thrombin-binding aptamer (Comp apt, Table S1) was added to the sample solutions for isolation of the cross-linked thrombin from the DNA scaffold. DNase I was then added to the sample solution for degradation of the DNA scaffold and the sample was applied to a SEC column. Fractions of high molecular weights for thrombin dimer and trimer were observed on the SEC chart (Fig. 2(B)). SDS-PAGE results of each fraction (inset of Fig. 2(B)) revealed that the apparent molecular weights of the cross-linked thrombin were c.a. 37.1 kDa, 67.4 kDa, and 114 kDa, respectively. These molecular weights corresponded to the estimated molecular weights of the cross-linked thrombin. The yields of dimerization and trimerization were 37% and 11%, respectively (Fig. S2).

The above investigations demonstrate the successful cross-linkage of thrombin molecules according to the DNA template. One of our objectives in this study was to produce a functional molecular device composed of plural protein molecules. We examined the activity of the cross-linked thrombin and found that the activity was maintained after the cross-linkage (Fig. S3).

The position of thrombin molecules on the DNA scaffold would affect the efficiency of the cross-linkage. We investigated the dimerization reactivity of thrombin molecules at the different immobilization domains of thrombin on the DNA scaffold. Fig. 3 shows the non-reducing SDS-PAGE results and the schemes of the different immobilization domains of thrombin on the DNA scaffold. No band for

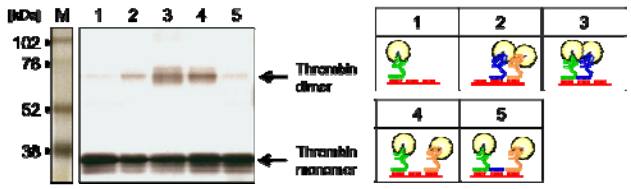


Fig. 3 Thrombin dimerization by varying the immobilization position of thrombin molecules on the DNA scaffold using BS3 (10-fold molar excess to thrombin). Variation of the different immobilization positions was as illustrated. Non-reducing SDS-PAGE (10%) was used. Gel was stained using the silver stain kit.

thrombin dimer was observed in the case of a single thrombin molecule on the DNA scaffold (lane 1). Dimerization of thrombin occurred in the presence of two thrombin molecules on the DNA scaffold (lanes 2–5). Interestingly, the dimerization efficiencies of thrombin molecules varied among lanes 2–5. In particular, lanes 3 and 4 showed relatively high efficiency. We thought that the reason for the different efficiencies was the rigidity and flexibility of the single-stranded and double-stranded oligonucleotides.[4a,8e] When there was a single-stranded region between two thrombin molecules (lane 4), the dimerization efficiency was higher than that of lane 5, probably due to the flexibility of the single-stranded part in the A2 domain. On the other hand, when A2 comp (A2 sequence without the aptamer sequence) was hybridized with the DNA scaffold, thrombin molecules were minimally cross-linked (lane 5). This was probably due to the rigidity of the double-stranded form at the A2 domain and to the long distance between the thrombin molecules.

In conclusion, we have demonstrated that DNA oligonucleotides work as scaffolds for the programmable protein-protein conjugation. To date, gene manipulation is often used for the creation of genetically conjugated proteins. However, there are limitations to producing conjugated proteins (e.g., protein with a huge molecular weight) using genetically modified cells. This methodology would have great potential to create a new class of functionally-integrated protein-protein conjugates because native wild-type proteins are suitable reagents for our methodology. However, the conjugation efficiency was still low. The possible reason, we thought, was due to the intramolecular cross-linkage in a thrombin molecule rather than the intermolecular cross-linkage between the neighboring thrombin molecules on the DNA scaffold. In future, the protein conjugateion efficiency would be improved using a suitable cross-linker or a site-specific linkage chemistry. This methodology can be expanded for constructing not only homo-protein conjugation but also hetero-protein conjugation by using different aptamer sequences, producing a novel protein conjugate with integrated multifunctions, and can be useful for the analysis of protein functions on protein chips or for protein carriers in the delivery of protein drugs.[17]

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Notes and references

[a] *Department of Applied Chemistry, Graduate School of Engineering, Kyushu University, 744 Moto-oka, Fukuoka 819-0395, Japan.Fax & Tel: +81-(0)92-802-2810*

[b] *Department of Chemical Science and Engineering, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe 657-850,(Japan. Fax: +81-(0)78-803-6070*

[c] *Center for future chemistry, Center for Future Chemistry, Kyushu University, 744 Moto-oka, Fukuoka 819-0395, Japan.Fax & Tel: +81-(0)92-802-2810*

Email: tmarutcm@crystal.kobe-u.ac.jp (TM), m-goto@mail.cstm.kyushu-u.ac.jp (MG).

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Electronic Supplementary Information

Programmable protein-protein conjugation via DNA-based self-assembly

Josui Shimada,[a] Tatsuo Maruyama,[b,*] Momoko Kitaoka,[a] Hisao Yoshinaga,[a] Koji Nakano,[a] Noriho Kamiya,[a,c] and Masahiro Goto [a,c*]

[a] Department of Applied Chemistry, Graduate School of Engineering, Kyushu University,

[b] Department of Chemical Science and Engineering, Kobe University,

[c] Center for Future Chemistry, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan

E-mail: tmarutcm@crystal.kobe-u.ac.jp (TM), m-goto@mail.cstm.kyushu-u.ac.jp (MG)

Experimental section

Materials

The oligonucleotides were obtained from Genenet Co. Ltd. (Fukuoka, Japan). Thrombin generation chromogenic substrate (β-Ala-Gly-Arg *p*-nitroanilide diacetate) was purchased from Sigma-Aldrich (St Louis, MO). Thrombin from human plasma, high activity was purchased from Merck Co., Bis (sulfosuccinimidyl) suberate (BS3) was purchased from Thermo Scientific Pierce Protein Research Products (Rockville, IL).

Table S1. DNA sequences used in the study.

Name	Length	Sequence
template DNA	42 mer	5'- GCT GAG TCT GCG TTG GTA CGC GGT GTT TCC AGG CAG CGA GTT -3'
A1	32 mer	5'- *CGC AGA CTC AGC* TTT TTG GTT GGT GTG GTT GG -3'
A2	32 mer	5'- *ACA CCG CGT ACC* TTT TTG GTT GGT GTG GTT GG -3'
A2 comp	12 mer	5'- *ACA CCG CGT ACC* -3'
A3	32 mer	5'- *CTC GCT GCC TGG* TTT TTG GTT GGT GTG GTT GG -3'
Comp apt	15 mer	5' – CCA ACC ACA CCA ACC – 3'

* Italic: hybridization sequence, underline: thrombin-binding aptamer sequence

Preparation of DNA-thrombin complex

For the preparation of the DNA scaffold, oligonucleotides (template, A1, A2, and A3) (1 μM) were mixed with a buffer (20 mM HEPES, 100 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, pH 7.9). The mixture was heated to 95 °C, incubated for 3 min, and cooled slowly to 4 °C at a decrease rate of -1.0 °C/min. The DNA scaffolds (1.5 μM) were mixed with thrombin (4.5 μM) and incubated for 1 h at room temperature. After adding 6 × loading buffer (Takara Bio Inc.), the samples were loaded on a 10% native polyacrylamide gel in TBE^+ buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, 5 mM $MgCl_2$ and 5 mM KCl). 10 bp DNA ladder (invitrogen) was used as a molecular marker. Electrophoresis was performed for 150 min at 4 °C at a constant 42 mA. After the electrophoresis, the gel was stained with SYBR Green II (Takara Bio Inc.) for 30 min. The gel was scanned using a transilluminator at 254 nm.

DNA-templated covalent cross-linking of thrombin

The DNA scaffolds (1.5 μM) were mixed with thrombin (4.5 μM) in a buffer solution as described above. BS3 was added in 50-fold molar excess of thrombin to the DNA-thrombin complex solutions for the covalent cross-linking of thrombin. After incubation for 1 h at room temperature, Tris-HCl buffer (final concentration; 100 mM, pH 8.0) was added to stop the cross-linking reaction. To analyze the linkage of thrombin, the samples were loaded on a 7.5% non-reducing polyacrylamide gel. Electrophoresis was performed for 30 min at a constant 42 mA. The gels were stained using a silver stain kit (Wako).

Confirmation of the multi-linking thrombin by MALDI-TOF-MS

After the DNA-templated cross-linkage of thrombin as described above, DNase I (2 U) was added to the reaction mixture. After the incubation for 1 h at room temperature, the samples were preconcentrated and separated from the DNase I and non-linked thrombin using Amicon® Ultra Centrifugal Filters (50kDa cutoff, Millipore). Then, the samples were concentrated, deionized using Zip Tip C4 (Millipore), and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (AutoflexIII, Bruker) using α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix.

Size-exclusion chromatography of the cross-linked thrombin

After the DNA-templated cross-linkage of thrombin, a complementary strand of thrombin aptamer (9 μM) was added to the reaction mixture. After the incubation for 1 h at room temperature, DNase I (2 U) was added to the reaction mixture. An hour later, the samples were purified and separated from DNA fragments using Amicon® Ultra Centrifugal Filters (30kDa cutoff) and diluted up to 100 μL with degassed TBS buffer (25 mM Tris-HCl, 137 mM NaCl, 2.68 mM KCl, pH 7.4). The sample solutions, containing thrombin monomer, dimer, and trimer, were applied to the size exclusion

chromatography (SEC) column (Superdex 200 10/ 300 GL, GE Healthcare). SEC was carried out using a BioLogic DuoFlow™ chromatography system and BioFrac™ Fraction Collector (Bio-Rad Laboratories, Inc. Hercules, CA) at a flow rate of 0.25 mL/min. The SEC elution profile at 280 nm was analyzed and Gaussian peak deconvolution of the SEC chart was performed using a software of IGOR Pro 6.0 (Oregon, USA). The peak areas of the cross-linked thrombin were integrated to calculate the yield of each cross-linked thrombin.

Assay of thrombin activity

The thrombin activity was measured with a thrombin substrate (0.5mM, thrombin generation chromogenic substrate) in TBS buffer containing 5mM $CaCl_2$ at 405 nm at 37 °C for 10 min.

Supplementary figures

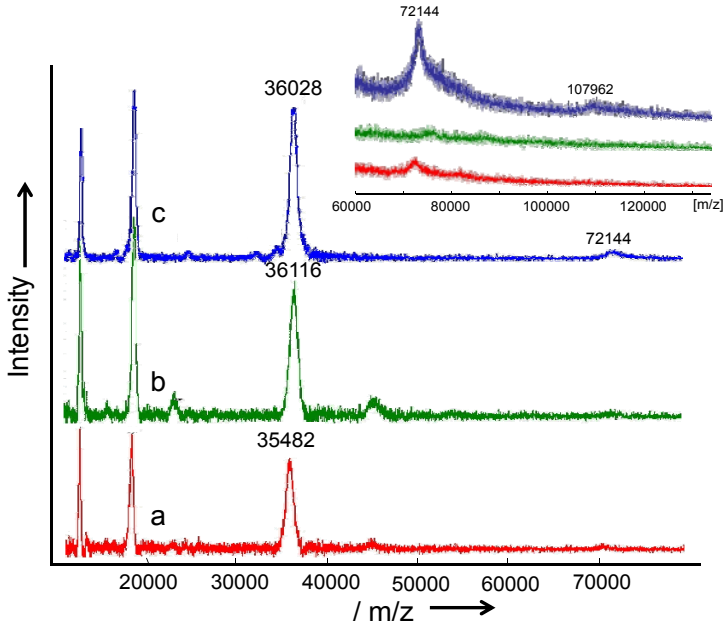

Fig. S1 MALDI-TOF-MS results of thrombin. (a) thrombin-only without BS3 and DNA scaffold, (b) thrombin with BS3 in the absence of the DNA scaffold, (c) thrombin with BS3 in the presence of the DNA scaffold and purification using 50 kDa MWCO membrane to remove thrombin monomer. All samples were desalted using ZipTip C4.

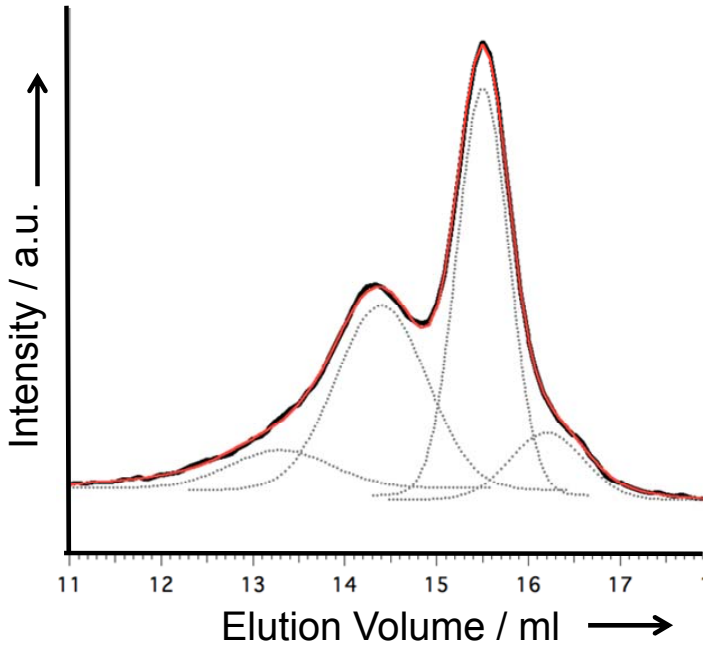

Fig. S2 Gaussian peak deconvolution of SEC chart of the cross-linked thrombin. The original chart of the cross-linked thrombin represented black line and the resultant fitted curve represented red line. The gray dashed line represents gaussian peaks of the cross-linked thrombin by IGOR Pro 6.0. Each peak was assigned as thrombin monomer or oligomers to calculate their yields.

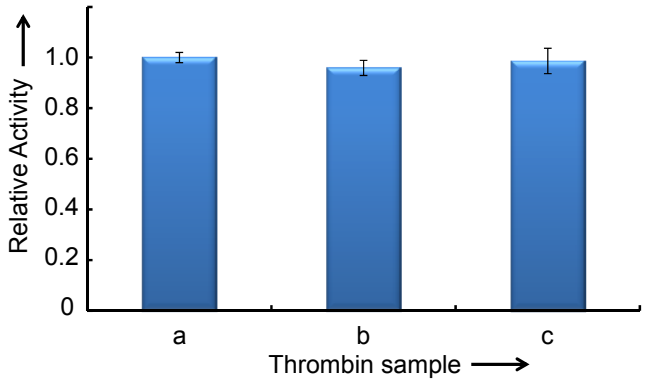

Fig. S3 Relative enzyme activities of native wild-type thrombin (a), cross-linked thrombin which was prepared in the absence of DNA scaffold (b), cross-linked thrombin which was prepared in the presence of DNA scaffold and purified using 50 kDa MWCO membrane to remove thrombin monomer (c). Each solution contained 0.148 μg/ml thrombin. The relative enzyme activities to the native wild-type thrombin were shown.