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A PNA–DNA hybridization chip approach for the detection of β-secretase activity

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Abstract—Developed was the addressable chip technology based on the PNA–DNA complementary hybridization equipped with short seven-mer PNA-encoded peptides that can be a versatile scaffold to monitor on-chip immunoassays. We also developed and validated a methodology to perform β-secretase enzyme assay with a highly sensitive fashion, resulting that a peptide substrate tethering dual fluorescent probes allowed us to detect β-secretase activity 10 times more sensitively than assays in solution.
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After successful completion of human genome sequencing, it becomes important to uncover roles of a number of proteins in a high-throughput fashion. Microarray/chip technologies have been developed as a powerful tool to analyze biological contents such as antibodies, proteins, and enzymes in various formats.[1–3] As one of the prominent chip approaches to proteomic studies, the PNA (peptide nucleic acid)–DNA complementary hybridization platform has been developed to analyze protein functions on the basis of addressable introductions of molecules of interest onto tagged positions.[4–6] PNA molecules were developed as DNA analogs having the capacity to form duplexes with complementary DNA sequences. The PNA–DNA hybridization chip has potentials such as allowance for (i) solid-phase synthesis of PNA-encoded peptides, (ii) biological events in solution phase, (iii) concentrations of probing molecules within a tiny area on the chip, and (iv) use of instrumentations developed for a DNA chip technology. These features meet the criteria for highly sensitive and reproducible measurements.

On the other hand, in Alzheimer's disease (AD) pathogenesis, amyloid β-peptide ($A\beta_{40}$ or $A\beta_{42}$) produced by β- and γ-secretases plays a critical role.[7] From the viewpoint of drug development, discovering inhibitors for an aspartic protease BACE1 identified as β-secretase is one of the most promising approaches because BACE1-knockout mice did not have significant phenotype.[8] So far, an excellent BACE1 assay system involving fluorescence measurements in solution has been commercially available for facilitating the screening of potent substrates or inhibitors.[9] Although such a homogeneous measurement is quite sensitive, relatively a large volume of the assaying mixture (~30 μL/well) is required, resulting in lacking a miniaturized and parallelized fashion. Therefore, the use of PNA-encoded peptides as an enzyme substrate (or inhibitor) seems to be still an exciting challenge because the PNA–DNA hybridization chip approach enables a treatment of multiplexed PNA-encoded peptide substrates with the enzyme of interest in solution, followed by an addressable introduction of the substrate PNAs onto an oligoDNA-modified chip, affording numerous information within a single experiment. We, herein, report the development of the BACE1 assay system utilizing the PNA–DNA hybridization chip technique (Fig. 1) toward the addressable chip format for a high-throughput analysis in proteomic studies.

The PNA regions that hybridize with DNA oligomers immobilized onto the solid surfaces were designed to comprise only seven bases including three adenine-PNAs and four cytosine-PNAs to facilitate productions of PNAs, which are mostly a half-length of the 12-mer[4a,b] or 14-mer PNA regions[4c,d] reported previously. To explore PNA–DNA duplex formations suitable for enzyme assay conditions, we performed the validation of such short PNA sequences in a chip

Keywords: PNA–DNA hybridization; Addressable chip; β-Secretase; On-chip assay; Immunoassay.
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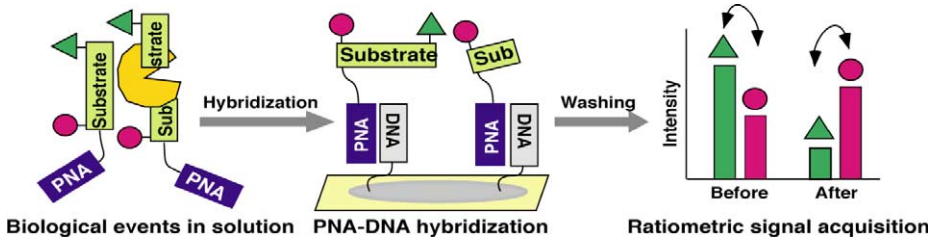

Figure 1. Schematic representation of enzyme assays on a chip format of the PNA–DNA complementary hybridization.

format. For accomplishment of this format, we designed and synthesized four different epitope tag-conjugated PNAs (T7-PNA1, c-Myc-PNA2, HA-PNA3, and FLAG-PNA4), in which the epitope tags were attached at the N-termini of the PNA sequences to form duplexes with DNA1, DNA2, DNA3, and DNA4 with different sequences, respectively (Fig. 2). Then, we examined immunoassays between the PNA conjugates and corresponding antibodies [anti-T7 (rabbit IgG, Bethyl Laboratory), anti-c-Myc (mouse IgG, Sigma), anti-HA (rabbit IgG, Sigma), and anti-FLAG (mouse IgG, Sigma)]. Additionally, ROX-PNA4 and TAMRA-PNA5, which have the dye moiety at the N-termini of the PNAs, were also designed to monitor hybridization efficiency depending upon PNA sequences. For BACE1 assay, the EVNLDAEF sequence, a Swedish mutant of β-secretase cleavage site in amyloid precursor protein (APP), was joined at the N-terminus of the PNA1 sequence, and TAMRA and ROX were attached at the N- and C-termini of the peptide sequence through lysine side chains, respectively. The use of dual fluorescent probes enables us to assay both N-type and C-type protease activities in the chip format different from systems with a single probe.[3b,c,4c] All PNA conjugates were synthesized by solid-phase peptide synthesis methodology, purified by HPLC, and characterized by MALDI-TOFMS. While DNAs immobilized onto the solid surfaces were comprised of 14 bases, 5′ half of which is a spacer region and 3′ half for a complementary hybridization region to PNA. The 5′-termini of the DNAs were modified with an amino group to form a covalent bond through the succinimidyl-functionalized glass slide (Geneslide, Toyo Kohan Co., Ltd, Japan).[10]

First, in order to examine specificities of the PNA–DNA hybridization, immunoassays were performed with the epitope tag-PNA conjugates on the glass slide modified with DNAs1, 2, 3, and 4 (2 mm in diameter/DNA spot) (Fig. 3).[11] Each assaying cocktail (20 μL) containing all of the four different PNAs, one of four antibodies corresponding to each complementary PNA conjugate, and secondary antibodies [both Cy3-labeled anti-mouse antibody (goat IgG, Amersham) and Cy5-labeled anti-rabbit antibody (goat IgG, Amersham)] was spotted onto the slide (Fig. 3, solutions 1–4). Anti-T7 and anti-HA were successfully detected through the Cy5 channel and also anti-c-Myc through the Cy3 channel. Unfortunately, only anti-FLAG antibody was not detected by the present technique. The assaying cocktail containing four epitope-tagged PNAs and all kinds of the corresponding antibodies and two different secondary antibodies (under the mixed condition of four anti-epitope tag antibodies) provided quite similar distributions of fluorescence signals on the slide (Fig. 3, solution 5).

DNA1	3′ GGTGGTT-TTTTTTT-NH$_2$ 5′
DNA2	GGTGTGT-TTTTTTT-NH$_2$
DNA3	GGTTGGT-TTTTTTT-NH$_2$
DNA4	TGGTGTG-TTTTTTT-NH$_2$
DNA5	GGTTTGG-TTTTTTT-NH$_2$
T7-PNA1	N H-MASMTGGQQMG-βAβA-ccaccaa-K-NH$_2$ C
c-Myc-PNA2	H-EQKLISEEDL-βAβA-ccacaca-K-NH$_2$
HA-PNA3	H-YPYDVPDYA-βAβA-ccaacca-K-NH$_2$
FLAG-PNA4	H-DYKDDDDK-βAβA-accacac-K-NH$_2$
ROX-PNA4	ROX-accacac-K-NH$_2$
TAMRA-PNA5	TAMRA-ccaaacc-K-NH$_2$
BACE-PNA1	N TAMRA-βA-EVNLDAEF-βA-K(ROX)-βA-ccaccaa-K-NH$_2$ C

Figure 2. Sequences of DNAs and PNAs designed in the present study. β-Secretase can cleave the amide bond between leucine and aspartic acid residues in the BACE-PNA1 sequence. 5(6)-ROX for ROX-PNA4, 5(6)-TAMRA for TAMRA-PNA5, and 5-TAMRA/5-ROX for BACE-PNA1 were employed in the sequences. Abbreviations: a, adenine-PNA monomer; c, cytosine-PNA monomer; TAMRA, 5(6)-carboxytetramethylrhodamine; ROX, 5(6)-carboxy-X-rhodamine; βA, β-alanine.

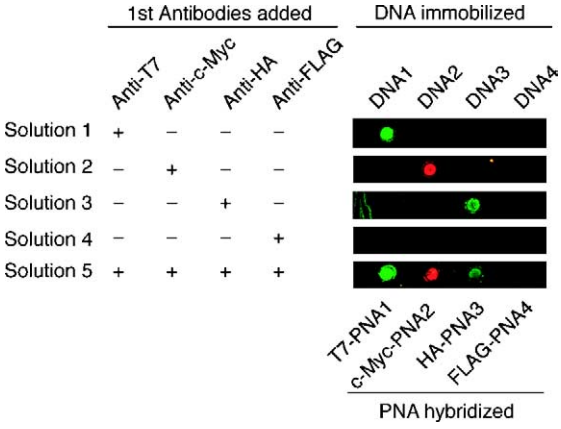

Figure 3. Fluorescence images of immunoassays performed on the PNA–DNA hybridization glass slides.[11] The emissions from Cy5 and Cy3 are expressed in green (λ_{ex} = 633 nm, λ_{em} = 670 nm) and red (λ_{ex} = 543 nm, λ_{em} = 570 nm), respectively. Each solution contains both Cy5-labeled anti-rabbit antibody and Cy3-labeled anti-mouse antibody. All of the immunoassays described in this figure were performed repeatedly on the same DNA-modified glass slide, suggesting that it is reusable.

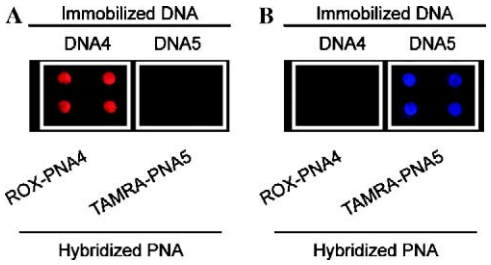

Figure 4. Fluorescence images of addressable immobilization of ROX-PNA4 (red) and TAMRA-PNA5 (blue) onto the DNA-modified slide through ROX channel (λ_{ex} = 633 nm, λ_{em} = 670 nm) (A) and TAMRA channel (λ_{ex} = 543 nm, λ_{em} = 570 nm) (B). A mixture (20 μL) of ROX-PNA4 (0.1 μM) and TAMRA-PNA5 (0.1 μM) was hybridized with DNA-modified slide according to the general procedure described in the Supporting Information.[11] A set of four spots in the same plate represent experiments performed under the same reaction condition for reproducibility.

These findings probably suggest that a lower melting temperature, T_m = 29.3 °C, for PNA4–DNA4 duplex affected hybridization efficiency compared with others with T_m = 31.0 °C for PNA1–DNA1 and PNA2–DNA2, and T_m = 30.2 °C for PNA3–DNA3, in which the T_m values were estimated as DNA–DNA duplexes calculated with parameters of the nearest-neighbor base pairs.[12] To examine hybridization efficiencies of PNAs with relatively lower T_m values, we performed the addressable immobilization of ROX-PNA4 (T_m = 29.3 °C) and TAMRA-PNA5 (T_m = 28.4 °C) onto the DNA-modified slide (Fig. 4). Even with lower T_m values, both ROX-PNA4–DNA4 and TAMRA-PNA5–DNA5 duplexes were successfully detected on the slide. Thus, in the case of FLAG-PNA4 immobilization onto the slide, antibodies associated with the epitope-tag-PNA4 with a lower T_m value might affect the complementary hybridization between FLAG-PNA4–DNA4 on the surface. Although the design of peptide–PNA conjugates must be more sophisticated, these results indicated that even short PNA regions containing seven bases formed duplexes stable enough to address complexes to each tagged position and encouraged us to employ the PNA1 sequence to perform the BACE1 enzyme assay on the present platform.

BACE-PNA1, a substrate-modified PNA which has two fluorescent molecules (TAMRA and ROX) suitable for a ratiometric analysis with excitation and emission bands at a longer wavelength, was designed to detect recombinant human BACE1 (rhBACE1, R&D Systems) activity (Fig. 2). The rhBACE1 activity was assayed both in solution and on-chip formats using the BACE-PNA1 as a substrate.[11] In solution assay (reaction volume 100 μL), the broad fluorescence band around 600 nm [overlapping with emissions both from TAMRA (580 nm) and ROX (610 nm)] was increased as a function of time and the ratio of TAMRA/ROX in fluorescence intensity reached a plateau at 10 h after the reaction started (Fig. 5). The concentration dependence of rhBACE1 in the hydrolytic activity was also examined in the solution assay, showing the lowest limitation of detection (LLD) to be around 100 nM (Fig. 5, inset).

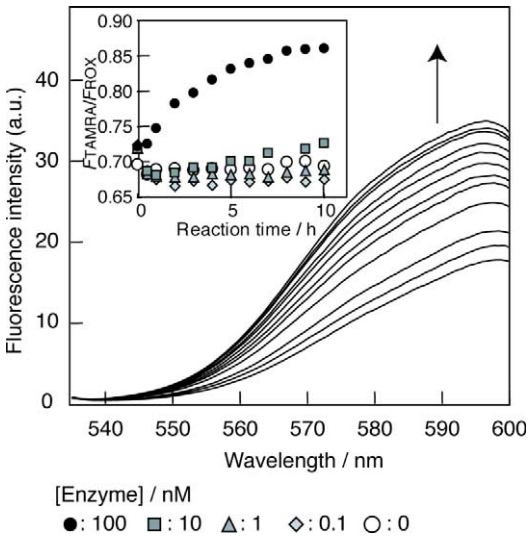

Figure 5. Fluorescence spectra of the rhBACE1 assay mixture at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h after the reaction started in 0.1 M AcOH/AcONa buffer (pH 4.5) at 37 °C ([rhBACE1] = 100 nM, λ_{ex} = 525 nm, λ_{em} = 535–600 nm, reaction volume 100 μL).[11] Inset shows changes in fluorescence intensity ratio [F_{TAMRA}(583 nm)/F_{ROX}(599 nm)] as a function of elapsed time varying upon rhBACE1 concentration ranging from 0.1 to 100 nM, indicating that the lowest limitation of detection is around 100 nM.

While, on the assay utilizing the PNA–DNA hybridization chip (2 mm in diameter/DNA spot), the increase in ROX and the decrease in TAMRA fluorescence intensi-

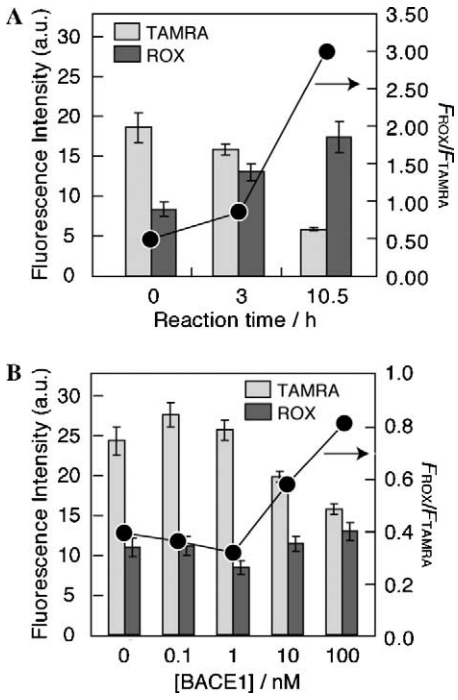

Figure 6. Changes in fluorescence intensity of TAMRA (λ_{ex} = 543 nm, λ_{em} = 570 nm) and ROX (λ_{ex} = 594 nm, λ_{em} = 614 nm) (bars, left *y*-axis) and in fluorescence intensity ratio [F_{TAMRA}(570 nm)/F_{ROX}(614 nm)] (closed circles, right *y*-axis) in the PNA–DNA hybridization chip format (2 mm in diameter/DNA spot).[11] (A) rhBACE1 activity (at 37 °C) depending upon reaction time, [rhBACE1] = 100 nM, and (B) upon rhBACE1 concentration ranging from 0 to 100 nM at 3 h.

ties were observed as a function of time, causing a significant elevation in the ratio of ROX/TAMRA due to which the TAMRA-containing fragments were released during the reaction then washed out, and the duplex formation between the fragmented PNA-encoded peptide and DNA was stabilized more than that between the full-length peptide–PNA and DNA on the surface (Fig. 6A).[11] Average of the ROX/TAMRA ratios at 10.5 h from five different spots within the same slide was 3.1 ± 0.1 (SD), indicating that the present chip approach provided highly reproducible results. The rhBACE1 concentration dependence in the enzyme assay was also examined on the PNA–DNA hybridization chip and afforded the LLD to be around 10 nM at 3 h after the reaction started (Fig. 6B). These results suggest that the platform of the PNA–DNA hybridization chip is approximately 10 times more sensitive than assays in solution, especially, washing out the released TAMRA-containing fragment followed by concentration of the residual PNA molecules within a tiny area on the chip is effective for such reproducible measurements.

In conclusion, we have demonstrated that seven-mer PNA-encoded peptides can be a versatile scaffold to monitor on-chip immunoassays, in which anti-epitope-tag antibodies were successfully addressed to the appropriate positions on the chip. We have also developed and validated a methodology to perform rhBACE1 enzyme assay with a highly sensitive fashion. The addressable chip approach would require only a small volume of sample solutions and provide high-throughput analyses and reproducible results in biological and diagnostic researches. The present platform can be applied to screening of potent substrates and inhibitors for rhBACE1.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.10.064.

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