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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.

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

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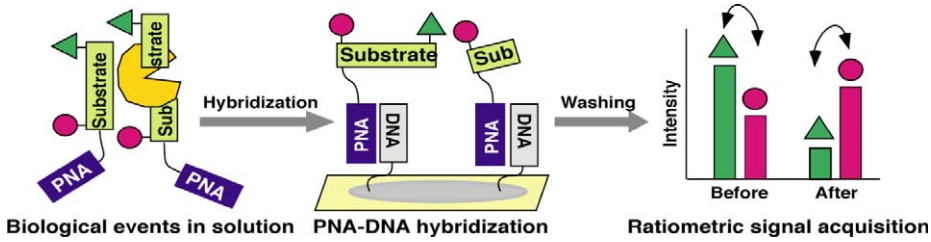

Figure 1. Schematic representation of enzyme assays on a chip format of the PNA–DNA complementary hybridization.

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.

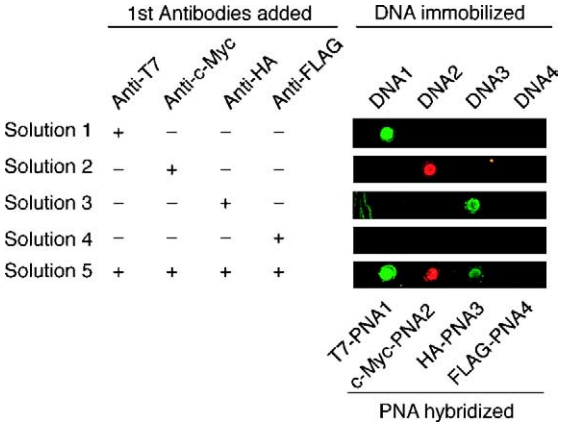

Figure 3. Fluorescence images of immunoassays performed on the PNA–DNA hybridization glass slides.[11] The emissions from Cy5 and Cy3 are expressed in green (λ_{ex} = 633 nm, λ_{em} = 670 nm) and red (λ_{ex} = 543 nm, λ_{em} = 570 nm), respectively. Each solution contains both Cy5-labeled anti-rabbit 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.