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A conjugated polymer–peptide hybrid system for prostate-specific antigen (PSA) detection†

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We developed fast and readily applicable microarray chips to detect PSA by designing a novel conjugated polymer (energy donor) and combining it with on-chip peptide synthesis. The selective cleavage of a probing peptide labelled with a dye or a quencher (energy acceptor) produced a fluorescence sensory signal *via* fluorescent energy resonance transfer (FRET).

Proteomics involving the large-scale study of proteins, particularly a comprehensive view of the structure, function and regulation of biological systems have formed the core technologies for protein analysis. Such strategies provide the development of specific protein biomarkers for disease diagnostics.[1] The on-chip peptide synthesis in proteomics may allow fast screening of spatially addressable peptide libraries in order to meet the increasing demand of simultaneous detection of a diverse range of biomolecular interactions and sequence specific bioactivity.[2] Most current methods for biomarker detection require target labelling, which is a costly and time-consuming process and requires subsequent stringent purification after the labelling. For example, commonly used separation and detection techniques are highly quantitative electrophoresis, high-pressure liquid chromatography (HPLC) separation, mass spectroscopy, enzyme linked immunosorbent assay (ELISA), and blotting techniques. These fastidious analysis methods make it difficult to perform real-time organism detection and fast diagnosis of cancer-related diseases.

Prostate cancer is a disease in which the cancer develops in the prostate, a gland in the male reproductive system, and has become the most commonly diagnosed cancer in men.[3] Even though it can be treated using cryosurgery, radiation therapy, androgen deprivation therapy, chemotherapy, and proton therapy, currently there is no effective treatment that significantly prolongs the life expectancy of a prostate cancer patient. Therefore, an early detection is critically important to save the life of prostate cancer patients. Clinically, diagnosis of prostate cancer was first conducted by measuring the concentration of a protein called prostate specific antigen (PSA) in the blood.[4]

Conjugated polymers are promising active materials to devise future generations of biosensors for the detection of biological molecules due to their molecular environment sensitivity and signal amplifying properties.[5] Many recent papers have reported the use of amplified fluorescence signal of conjugated polymers as a sensitive signal reporter for the detection of a number of biological analytes.[6] Upon binding with a target biological molecule, conjugated polymer-based sensory systems showed a remarkable change in fluorescence emission by means of efficient electron transfer or fluorescence resonance energy transfer (FRET).[7] Herein, we report label-free and sensitive protein chips using a highly fluorescent conjugated polymer to detect PSA. We identified a hexapeptide molecule which can be enzymatically cleaved by PSA and directly synthesized the peptide on a monolayer of an amine-functionalized conjugated poly(oxadiazole) (P1). After parallel peptide synthesis on the P1-coated slide followed by labelling with a quencher or a dye, the slide was incubated with a PSA solution to observe FRET-induced signal-amplified fluorescence change upon peptide cleavage (Fig. 1).

Two strategies were employed in this study as shown in Fig. 1. In the first, Strategy I, a quencher was incorporated at the end of the peptide sequence. As the quencher molecule we used DABCYL whose absorption has a good overlap with the polymer emission, satisfying the requirement for efficient FRET. The energy transfer from P1 to DABCYL will reduce the fluorescence emission intensity of P1. After the enzymatic cleavage of the peptide sequence by PSA, however, the fluorescence emission of P1 should be recovered due to the removal of the quencher from the peptide. In the second strategy, fluorescein instead of the quencher was tagged at the end of the peptide sequence. Fluorescein was chosen to establish an efficient FRET from the energy donor (P1) to the acceptor (fluorescein). The attachment of fluorescein at the end of the peptide grown from the P1-immobilized substrate will result in

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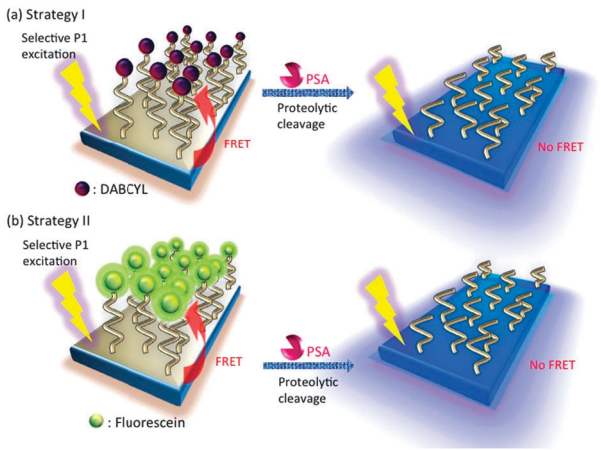

Fig. 1 PSA detection by conjugated polymer–peptide hybrid chips having (a) a quencher (Strategy I) and (b) a fluorescent dye (Strategy II).

emission quenching of P1 and amplified dye emission simultaneously. The selective cleavage of the peptide sequence by PSA will remove fluorescein, and as a consequence induce the recovery of the P1 emission and the suppression of the amplified dye emission. The same peptide without DABCYL or fluorescein on the P1-coated substrates was also prepared as a negative control.

Glass slides were dipped in ammonia–hydrogen peroxide cleaning solution at 80 °C for 30 min, followed by piranha solution for 15 min. The glass slides were further treated with aminopropyltrimethoxysilane (APTMS) to derive the amine functionality on the surface. SCM–PEG–Fmoc (Laysan Bio, Arab, AL) was reacted with the amino group of the APTMS to form a reactive linker for P1. The m-PEG chain was used to control the density of the amine functional groups on the surface by interspersion of the reactive amine group. We introduced the long PEG linker between the slide and the P1 to increase the accessibility of PSA to the peptide sequence and to reduce non-specific adsorption of PSA onto the substrate at the same time. As we were concerned about the limited accessibility of PSA to the surface-tethered peptide sequence, the long PEG linker later turned out to be necessary for a successful peptide cleavage by PSA. The details of the synthetic route and characteristic data of the conjugated polymer, poly(oxadiazole-*co*-phenylene-*co*-fluorene) (P1), have been previously reported.[7] The fluorene unit of P1 was introduced to increase polymer solubility in organic solvents and to facilitate solution-based film fabrication. After deblocking of Fmoc-amine by piperidine treatment, 1,4-phenylenediisothiocynate was then reacted with free amine to form a reactive isothiocyanato group, which was used as a linker for P1 tethering (P1 structure and slide preparation in detail are provided in the ESI†).

Standard Fmoc chemistry was used for all steps using Fmoc-protected amino acids, HATU, and DIEA in DMF using a peptide synthesizer. An *in situ* integrated parallel peptide synthesis using solid-phase peptide chemistry and photogenerated acid chemistry was conducted using an Expedite 8909 DNA synthesizer modified for peptide synthesis and equipped with an optical setup.[8] The amine groups on P1 were used to anchor P1 to the glass substrate and as the reaction group for the peptide synthesis at the same time. In each coupling step, unreacted sites were capped with acetic anhydride to prevent undesirable peptide sequences. The removal of the protecting groups of side chains at the end of the synthesis was achieved by incubating in a cleavage cocktail of trifluoromethanesulfonic acid, trifluoroacetic acid (TFA), dichloromethane, ethanedithiol, and thioanisole. It has been previously noted that P1 containing the oxadiazole unit is uniquely stable in UV and strong acid treatment therefore suitable for the on-chip oligonucleotide synthesis in the solid state.[9] Finally, 4,5-carboxyfluorescein (FAM, Molecular Probes, Grand Island, NY) was labelled at the end of the peptide chains by HOBT based-carbodiimide chemistry. DABCYL (4-{[4-(dimethylamino)-phenyl] azo}-benzoic acid, AnaSpec, Inc, Fremont, CA) coupling was performed in the same manner as FAM labeling. The PSA proteolysis test was conducted by adding a PSA solution onto the peptide-coated spots on the microarrays. Peptide chips were then incubated at 37 °C for 4 hours and the photoluminescence spectra of the slides were examined before and after the incubation (detailed descriptions for peptide synthesis are in the ESI†).

The hexapeptide sequence was identified from a previous study.[10] Several peptide sequences corresponding to the cleavage map for semenogelin-I and semenogelin-II have been proposed. PSA acts as a protease that catalyses hydrolysis of semenogelin I and II, gel-forming proteins in the human semen, in the seminal coagulum. The preferred subsite occupancy was previously defined for peptide cleavage by PSA using phage display and iterative optimization of native substrate sequences. Accordingly, the sequence QHY–SSN appears to have the highest cleavage rates (1.00 relative value, QRY–SSN: 0.01, see below) among all other hexapeptides and is therefore used in this study.

The fluorescence spectrum of the P1-coated peptide slides before and after the incubation with PSA was obtained from a photoluminescence spectrometer. First, in the first strategy having the DABCYL quencher, the fluorescence emission of P1 was significantly quenched by FRET from P1 to DABCYL before addition of PSA (Fig. 2). After the proteolytic cleavage of the hexapeptide sequence by PSA, the emission intensity of P1 was fully recovered. The negative control shown in Fig. 2b was made with the same hexapeptide sequence but without the attachment of DABCYL. No quenching of the P1 emission was observed of course and therefore there was no difference in the P1 emission intensity before and after the incubation with PSA. This result demonstrates that direct on-chip peptide synthesis onto the P1-coated glass slide and the cleavage of the hexapeptide substrate by PSA were successfully accomplished.

In the second approach, Strategy II, as illustrated in Fig. 1b, instead of the DABCYL quencher a fluorescein dye was attached to the end of the hexapeptide. Fig. 3 shows the emission spectra of P1 before and after the incubation with PSA. Before PSA treatment the discrete fluorescein emission peak was observed at 525 nm when P1 was selectively excited at 380 nm because the fluorescein emission was amplified through an effective FRET from P1 to fluorescein. Upon the release of the fluorescein dye when the hexapeptide was cleaved by PSA, the emission from fluorescein almost completely disappeared, whereas the P1 emission was restored. Our preliminary data showed that the detection limit of the P1-coated signal amplifying scheme was in the range of nanomolar concentration regime (Fig. S5, ESI†). This signal conversion from fluorescein and P1 clearly indicates

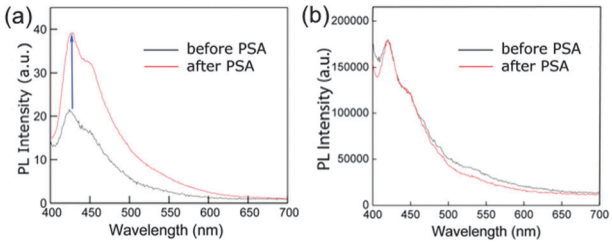

Fig. 2 Fluorescence spectra of the P1-coated peptide slides with (a) or without (b) DABCYL (Strategy I with a quencher) before and after PSA addition. Black and red lines indicate fluorescence signal before and after adding PSA, respectively.

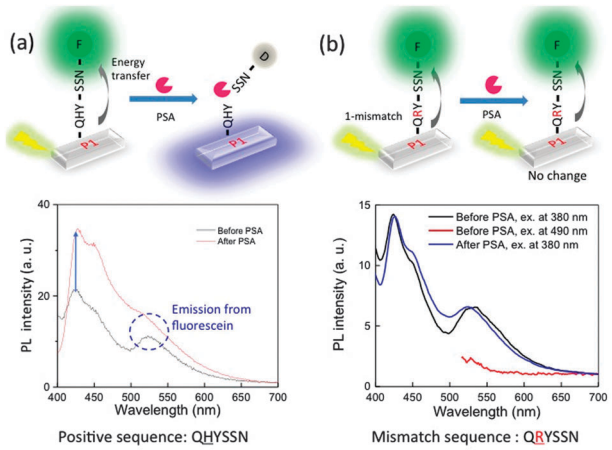

Fig. 3 Detection study using a fluorescein dye (Strategy II with a fluorescence acceptor). (a) Fluorescence energy was transferred from P1 to the dye (D) before PSA addition. After the addition of PSA to cleave the hexapeptide (QHY–SSN), the fluorescein emission disappeared and instead, P1 emission was fully recovered. The black and red lines indicate fluorescence before and after the addition of PSA respectively. (b) Selectivity test by using a 1-mismatching peptide sequence (QRY–SSN). The black and blue lines indicate fluorescence emission before and after the addition of PSA (excitation wavelength: 380 nm). The red line indicates the fluorescein emission upon direct excitation at 490 nm.

an efficient Förster energy transfer from P1 to fluorescein. The FRET efficiency calculated by the equation 1 − (PL intensity after PSA at 525 nm)/(PL before PSA at 525 nm) was 0.78 when the long PEG spacer was used, whereas it was only 0.2 when the PEG linker was not used. This confirms that the long and flexible PEG linker enables the hexapeptide probe to be fully accessible to PSA. We also checked the specificity of the hexapeptide sequence QHY–SSN toward the enzymatic cleavage of PSA by constructing a P1-coated peptide chip having one mismatch in the peptide sequence QRY–SSN as a negative control. This sequence was reported to have a much lower cleavage rate due to the perturbed electrostatic potential of the catalytic triad.[10] The results presented in Fig. 3b show no change in the P1 emission before and after the incubation with PSA due to the single-mismatch in the peptide sequence. In the same figure, direct excitation of fluorescein at 490 nm produced only much weaker fluorescence emission, confirming that the fluorescein emission was largely amplified by FRET from P1 to fluorescein when P1 was selectively excited at 380 nm.

We have demonstrated a label-free and signal amplifying peptide microarray using a conjugated polymer as a FRET donor and a DABCYL quencher or a fluorescein dye as a FRET acceptor. The FRET donor and the acceptor were connected through a hexapeptide sequence, QHY–SSN, for which PSA is a specific protease. The specific enzymatic cleavage of QHY–SSN sensitively prevented FRET between the polymer to the reporter and produced fluorescence recovery. A negative control having no reporter and a single-mismatch sequence of QRY–SSN, respectively, showed no signal generation, confirming the specificity of the peptide microarray for PSA detection. A long and flexible PEG linker turned out to be necessary to realize the protease activity of PSA toward the hexapeptide probe tethered to the P1-coated substrate. Conjugated polymer (P1)–peptide hybrid chips optimized for a particular target probe at low concentration may then be utilized as potent diagnostic kits in clinical use.

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