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Cleavage-induced fluorescence change via hydrophilicity control: A new strategy for biological application

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Abstract

In this work, we report a novel stimuli-sensitive probe working on the principle of the cleavage-induced fluorescent change via hydrophilicity control. In our Si–Py–Ac–PEG probe, cleavage of hydrophilic PEG moiety in acidic environment induces hydrophobic interaction of pyrenes to increase their excimer emission. Our fluorescent probe (Si–Py–Ac–PEG) was successfully applied to the spotting microarray and nanoparticle suspension to demonstrate the potential of biological applications, i.e. protein chip and bioimaging.

Keywords: Hydrophilicity control; Excimer emission; Cleavage; Microarray; Biomedical imaging

1. Introduction

Stimuli-responsive molecular probes have gained attractions for the potential applications in biotechnology such as bioimaging [1] and array-based molecular sensors [2]. In particular, interest in optical imaging probes producing different responses by detecting environmental changes such as biological materials [1,3] or ionic concentration [4] is growing for investigating bio-related phenomena. Molecular fluorescent probes displaying fluorescence resonance energy transfer (FRET) or excimer (or dimer) emission properties have been most intensively investigated by labeling the probes to a labile site exposed to the environmental changes [3]. However, attempts to apply fluorophores having the specific luminescent properties by intermolecular interaction are rather limited in the field of biotechnology due to the low water solubility of those fluorophores in physiological conditions. Improving water solubility of hydrophobic molecules is one of the important issues in pharmaceutical industries and other bio-related fields. One of the most promising method is chemical conjugation of poly(ethylene glycol) (PEG) to the water-insoluble molecules. PEG modification, so called PEGylation, is well known to alter solubility characteristics both in aqueous and organic solution as well as to increase the stability of the compounds in physiological conditions [5]. Such characteristics of PEG have extended the application of PEGylated micro- and nanostructures to biomedical field as delivery carriers of hydrophobic drugs and nanostructured biosensors [6]. For a typical nanostructure-based sensing system, optimization concerning blocking of unspecific interactions such as undesired self-aggregation is required for a best result. In various biofunctionalized nanostructures for different bio-assays, it is mostly demanded to realize biochip-based detection with its potential for parallelization of the sensing groups in a plate-like substrate [7]. Smart fluorescent probes containing solubilized (PEGylated) hydrophobic fluorophores either in solution based assay or biochip-based systems are believed to offer a broad spectrum of potential applications in diagnostics and to extend the limits of molecular diagnostics to a nanoscale detection.

In this work, we demonstrate a novel stimuli-sensitive sensor working on the principle of the cleavage-induced fluorescent

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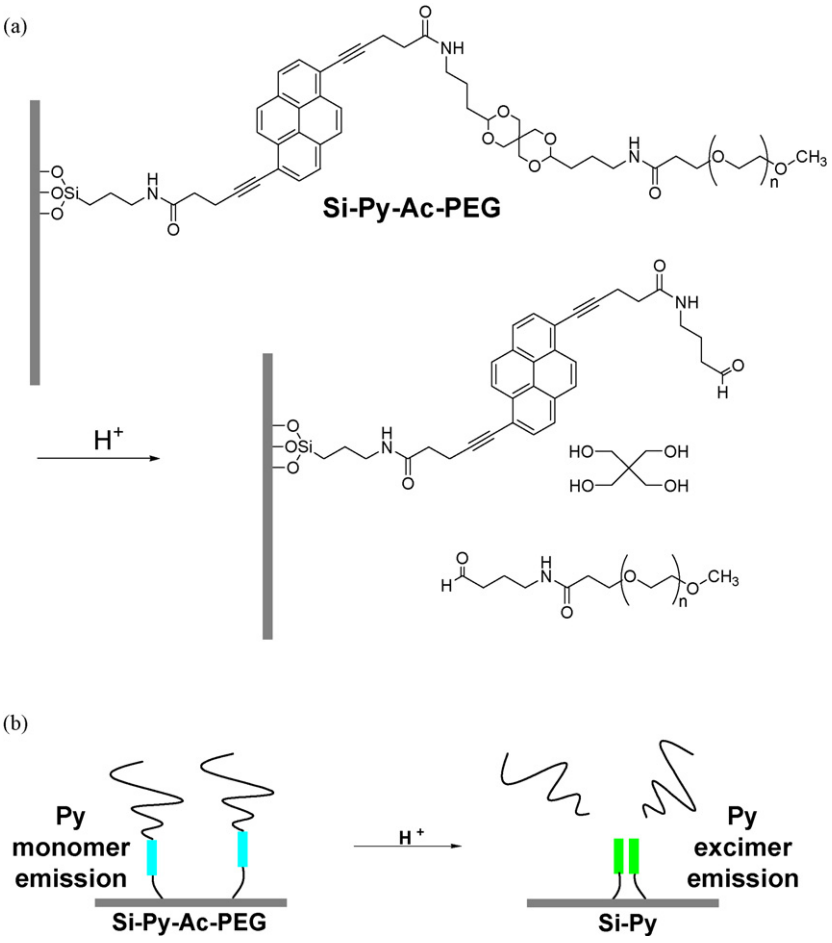

Fig. 1. (a) Acidic cleavage of Si–Py–Ac–PEG; (b) schematic representation of cleavage-induced fluorescence change.

change via hydrophilicity control. In the design of molecular probe, labile groups cleavable under acidic condition were introduced as a model recognition site to explore the possible application of this system to the sensors detecting the presence of specific enzymes (see the schematic representation of our strategy illustrated in Fig. 1).

2. Materials and methods

2.1. Instruments

^{1}H NMR measurements were recorded on a Avance DPX-300 (300 MHz, Bruker, Germany) and a Avance-600 (600 MHz, Bruker, Germany) in $CDCl_3$ or DMSO-d_6 solution. Mass spectra were measured on a JMS-AX505Wa (JEOL) by EI and a Voyager-DE^{TM} STR Biospectrometry Workstation (Applied Biosystems Inc.) by MALDI-TOF method. The data of elemental analysis was measured on EA1110 (CE Instrument, Italy). UV–vis absorption and fluorescence spectra were measured on a UV-1650PC (Shimadzu, Japan) and a RF-500 spectrofluorophotometer (Shimadzu, Japan), respectively. The microarrays were fabricated with a conventional microarrayer (Proteogen, Korea) and analyzed with a ArrayWorx biochip reader (Applied Precision, USA).

2.2. Synthesis

2.2.1. Synthesis of 1,6-dibromopyrene (2)

1,6-Dibromopyrene (**2**) was synthesized according to literature method [8]. Briefly, bromine (10 mL, 194.7 mmol) in carbon tetrachloride (500 mL) was added during 5 h with stirring to a solution of pyrene (20 g, 98.9 mmol) in carbon tetrachloride (500 mL). The precipitate was collected after 12 h and resolved by fractional crystallization from toluene (yield: 11.8 g, 33%). ^{1}H NMR (300 MHz, DMSO-d_6, 100 °C) δ [ppm]: 8.42 (d, J = 9.3 Hz, 2H), 8.39 (d, J = 8.3 Hz, 2H), 8.33 (d, J = 9.3 Hz, 2H), 8.27 (d, J = 8.3 Hz, 2H) MS [M+]: (EI) calcd. for $C_{16}H_8Br_2$, 360.4; found, 360. Anal. Calcd. for $C_{16}H_8Br_2$: C, 53.37; H, 2.24. Found: C, 53.12; H, 2.21.

2.2.2. Synthesis of dimethyl 5,5′-(pyrene-1,6-diyl)dipent-4-ynoate (5)

Methtyl 4-pentynoate (**4**) was synthesized according to literature method [9]. Briefly, a solution of 4-pentynoic acid (5 g, 50.9 mmol) and H_2SO_4 (3 g) in dry MeOH (100 mL, 2.4 mol) was heated under reflux for 12 h. The solution was extracted with CH_2Cl_2, and the CH_2Cl_2 extract was washed with a saturated $NaHCO_3$ solution and concentrated to give crude **4** (yield: 4.2 g, 73%). A solution of **2** (2 g, 5.5 mmol),

4 (1.9 g, 16.5 mmol), bis(triphenylphosphine) palladium(II) chloride (78 mg, 0.11 mmol), and copper iodide(I) (53 mg, 0.275 mmol) in dried triethylamine (80 mL) was refluxed under a nitrogen atmosphere for 30 h. The solution was extracted with dichloromethane, and the dichloromethane extract was dried and concentrated. The residue was purified by column chromatography on silica gel with chloroform to give **5** as a pale yellow solid (yield: 1.6 g, 68%) ^{1}H NMR (300 MHz, $CDCl_3$) δ [ppm]: 8.52 (d, J = 9.1 Hz, 2H), 8.04–8.10 (m, 4H), 3.78 (s, 6H), 2.98 (t, J = 7.6 Hz, 4H), 2.81 (t, J = 7.6 Hz, 4H). ^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm]: 171.981, 131.201, 130.210, 129.727, 128.170, 125.355, 125.284, 123.224, 118.168, 95.645, 79.289, 51.507, 32.867, 15.259. MS [M+]: (EI) Calcd. for $C_{28}H_{22}O_4$, 422.5; found, 422. Anal. Calcd. for $C_{28}H_{22}O_4$: C, 79.60; H, 5.25. Found: C, 79.91; H, 5.48.

2.2.3. Synthesis of bis(2,5-dioxopyrrolidin-1-yl) 5,5′-(pyrene-1,6-diyl)dipent-4-ynoate (7)

A mixture of **5** (1 g, 2.4 mmol), LiOH (0.8 g, 19.2 mmol), THF (75 mL), H_2O (45 mL) was stirred in an ice bath for 30 min. The solution was sonicated for 12 h at 50 °C and then extracted with ethylacetate. The water extract was acidified with HCl and the mixture was extracted with ethylacetate. The ethylacetate extract was washed with water and concentrated to give crude **6** (yield: 0.89 g, 95%). A solution of **6** (0.3 g, 0.76 mmol), EDC (0.58 g, 3 mmol) and *N*-hydroxysuccinimide (0.7 g, 6 mmol) in anhydrous DMF (30 mL) was stirred for 30 h at RT and extracted with ethylacetate. The ethylacetate extract was dried and concentrated. The residue was purified by column chromatography on silica gel with ethylacetate to give **7** as a yellow solid (yield: 355.2 mg, 80%). ^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm]: 8.52 (d, J = 9.1 Hz, 2H), 8.31 (d, J = 8.2 Hz, 2H), 8.28 (d, J = 9.1 Hz, 2H), 8.15 (d, J = 8.2 Hz, 2H), 3.21 (t, J = 6.2 Hz, 4H), 3.04 (t, J = 6.2 Hz, 4H), 2.87 (s, 8H). ^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm]: 70.069, 167.849, 131.265, 130.330, 129.921, 128.223, 125.721, 125.277, 123.203, 118.029, 94.466, 79.766, 30.011, 25.444, 14.992. MS [M+]: (MALDI-TOF (reflector mode)) Calcd. for $C_{34}H_{24}N_2O_8$, 588.56; found, 588.35. Anal. Calcd. for $C_{34}H_{24}N_2O_8$: C, 69.38; H, 4.11; N, 4.76. Found: C, 69.10; H, 4.29; N, 4.73.

2.2.4. Synthesis of trifluoro-N-[9-propanamino-2,4,8,10-tetraoxaspiro[5.5]undecyl] acetamide (12)

A three-neck round bottomed flask equipped with a dropping funnel and N_2 inlet–outlet was charged with TUDA (**10**) (3 g, 10.9 mmol) and triethylamine (0.76 mL, 5.5 mmol) in methanol (50 mL). The reaction mixture was cooled down to 0 °C in an ice bath. Trifluoroethylacetate (0.65 mL, 5.5 mmol) in methanol (10 mL) was added dropwise to the solution. After the addition was completed, the reaction mixture was warmed up to RT and stirred for 12 h. After methanol and trifluoro ethylacetate were distilled off under vacuum, the product was purified by column chromatography on silica gel with methanol to give **12** as a amber-colored liquid (yield: 1.58 g, 78%). ^{1}H NMR (300 MHz, $CDCl_3$) δ [ppm]: 7.00 (broad s, 1H, –CO**NH**–), 4.45–4.61 (m, 4H, –O–**CH**–O–, –**CH$_2$**–NH–), 3.29–3.42, 3.49–3.64 (m, m, 8H, –C–**CH$_2$**–O–), 2.70 (t, J = 7.4 Hz, 2H, –**CH$_2$**–NH_2), 1.54–1.77 (m, 8H, –CH–**CH$_2$**–**CH$_2$**–CH_2).

2.2.5. Synthesis of NH_2–Ac–PEG (14)

Triethylamine (4.7 mL, 33.75 mmol) was added dropwise to a stirred solution of **12** (1 g, 2.7 mmol) and mPEG-SPA (M_W: 2000, purchased from Nektar Therapeutics) (4.55 g, 2.25 mmol) in anhydrous chloroform (20 mL) under a nitrogen atmosphere. After 12 h stirring at RT, the solution was concentrated and the crude product was precipitated with cold diethyl ether to remove *N*-hydroxysuccinimide. The excess **12** was removed by column chromatography on silica gel with chloroform:methanol (10:1). The product was reprecipitated in diethyl ether to give **13** as a white solid (yield: 3.86 g, 75.3%). A solution of **13** (3.5 g, 1.5 mmol) in aq. 1N aq. NaOH (10 mL) was stirred at RT for 6 h. The solution was extracted with methylenechloride and the methylenechloride extract was dried with anhydrous magnesium sulfate. The solution was concentrated and precipitated in diethyl ether to give **14** as a white solid (yield: 2.94 g, 89.8%). ^{1}H NMR (300 MHz, $CDCl_3$) δ [ppm]: 6.55 (br s, 1H), 4.45–4.61 (m, 4H), 3.5–3.8 (m, 236H), 2.18–3.43 (m 7H), 3.2–3.3 (m, 4H), 2.75 (t, J = 7.0 Hz, 2H), 2.45 (t, J = 5.7 Hz, 2H), 1.5–1.7 (m, 8H). MS [M+]: (MALDI-TOF (chloroform, reflector mode)) 2452.

2.2.6. Synthesis of Py–PEG (8a)

Triethylamine (0.36 mL, 2.6 mmol) was added dropwise to a stirred solution of mPEG-NH_2 (M_W 2000, purchased from Sunbio Inc.) (113 mg, 56.6 μmol) and 7 (100 mg, 169.8 μmol) in anhydrous dimethylformamide (20 mL) under a nitrogen atmosphere. After a day stirring at RT, the solution was purified by dialysis against DMF using a Cellu. Sep membrane (Membrane Filteration Products Inc.) with a MWCO of 1000 for 4 days and the solution was dried under vacuum. The product was dissolved in methylenechloride and precipitated with cold diethyl ether to give **8a** as a yellow solid (yield: 85 mg, 62.3%). ^{1}H NMR (300 MHz, $CDCl_3$) δ [ppm]: 8.52 (d, J = 8.2 Hz, 1H), 8.49 (d, J = 8.8 Hz, 1H), 8.05–8.12 (m, 6H), 6.51 (br s, 1H), 3.5–3.9 (m, 190H), 3.41 (s, 3H), 3.01 (t, J = 7.3 Hz, 2H), 2.98 (t, J = 7.6 Hz, 2H), 3.01 (t, J = 6.8 Hz, 2H), 2.66 (t, J = 7.2 Hz, 2H). MS [M+]: (MALDI-TOF (water, linear mode)) 2465.

2.2.7. Synthesis of Py–Ac–PEG (8b)

This compound was obtained as a yellow solid (yield: 49 mg, 58.2%) from **14** (74 mg, 28 μmol) and **7** (50 mg, 85 μmol), using the procedure described for **8a**. ^{1}H NMR (600 MHz, $CDCl_3$) δ [ppm]: 8.50–8.55 (m, 2H), 8.05–8.11 (m, 6H), 6.51 (br t, 1H), 5.98 (br t, 1H), 4.18–4.43 (m, 6H), 3.5–3.8 (m, 236H), 2.18–3.43 (m 7H), 3.20–3.24 (m, 4H), 3.03–3.13 (m, 4H), 3.01 (t, J = 7.4 Hz, 2H), 2.61 (t, J = 7.0 Hz, 2H), 2.43 (t, J = 5.7 Hz, 2H), 1.5–1.7 (m, 8H). MS [M+]: (MALDI-TOF (chloroform, reflector mode)) 2898.

2.2.8. Synthesis of $(SiOEt)_3$–Py–PEG (9a)

A solution of **8a** (50 mg, 20.3 μmol) in anhydrous chloroform (20 mL) was stirred in ice bath under an argon atmosphere. One molar dicyclohexylcarbodiimide solution (27 μL, 27 μmol) was added dropwise to the solution and then the reaction mixture

was warmed up to RT 3-aminopropyltriethoxysilane (APTES) (7 μL, 30.5 μmol) was added to the stirred mixture at RT. After a day stirring at RT, the mixture was filtered to remove dicyclohexylurea and the solvent was concentrated. The product was dissolved in methylenechloride and precipitated three times with cold diethyl ether to give **8a** as a yellow solid (yield: 30 mg, 56.1%). ^{1}H NMR (300 MHz, $CDCl_3$) δ [ppm]: 8.52 (d, J = 9.0 Hz, 2H), 8.05–8.12 (m, 6H), 6.51 (br t, 1H), 6.11 (brt, 1H), 3.5–3.9 (m, 196H), 3.41 (s, 3H), 3.01 (t, J = 7.4 Hz, 4H), 2.66 (t, J = 7.4 Hz, 4H), 1.57–1.68 (m, 4H), 1.16 (t, J = 7.0 Hz, 9H), 0.67 (t, J = 7.3 Hz, 2H).

2.2.9. Synthesis of $(SiOEt)_3$–Py–Ac–PEG (**9b**)

This compound was obtained as a yellow solid (yield: 28 mg, 52.7%) from **8b** (50 mg, 17.3 μmol) and APTES (6 μL, 25.9 μmol), using the procedure described for **9a**. ^{1}H NMR (600 MHz, $CDCl_3$) δ [ppm]: 8.53 (m, 2H), 8.05–8.11 (m, 6H), 6.49 (br s, 1H), 6.15 (br s, 1H), 5.96 (br t, J = 5.3 Hz, 1H), 4.18–4.43 (m, 6H), 3.5–3.8 (m, 242H), 2.18–3.43 (m 7H), 3.20–3.24 (m, 4H), 3.02 (t, J = 7.2 Hz, 4H), 2.61 (t, J = 6.7 Hz, 4H), 2.43 (t, J = 5.6 Hz, 2H), 2.0–2.1 (m, 4H), 1.5–1.7 (m, 12H), 1.17 (t, J = 7.1 Hz, 9H), 0.65 (t, J = 7.2 Hz, 2H). MS [M+]: (MALDI-TOF (chloroform, reflector mode)) 3014.

2.3. Fabrication of microarray and fluorescence image analysis

A slide glass (76 mm × 24 mm) was cleaned with a concentrated 'Piranha' solution (70%, v/v H_2SO_4, 30%, v/v H_2O_2) and was thoroughly rinsed with deionized water and dried with nitrogen gas. The silane compounds (**9a** or **9b**, 1 mM) in DMF were spotted onto the slide glasss using conventional microarrayer (Proteogen, Republic of Korea). After spotting, the slide glass was dried for 24 h in 70% humidity to afford adsorption onto the glass and self-polymerization of silane groups. The spotted slide glass was rinsed three times with deionized water followed by blowing with nitrogen gas, and then was dropped with deionized water and 35% hydrochloric acid. After 1 h incubation at room temperature, the slide glass was then rinsed five times with deionized water and blowed with nitrogen gas. Thereby prepared fluorescent spots were analyzed by using the ArrayWorx biochip reader (Applied Precision, USA). The excitation wavelength was 350 nm and the emission slit was set to 520 nm.

2.4. Fabrication of nanoparticle probe and fluorescence measurement

Silica nanoparticles (SiNPs) with the monodisperse size of 150 nm were prepared by the well-stabilized Stöber and Fink reaction [10]. A mixture of nanoparticles suspension (1 mL of 30 mg/mL), **9b** (10 mg) and ammonium hydroxide (20 L) in anhydrous DMF (3 mL) was stirred under nitrogen atmosphere. After 8 h stirring at 70 °C, the PEGylated silica nanoparticles were isolated by centrifugation and washed five times with anhydrous DMF to remove unreacted **9b**. After solvent was changed to deionized water (1 mL), anhydrous lactose (5 mL) was added to the nanoparticles solution and the solution was freeze dried during 3 days. The prepared probes were redispersed in deionized water (3 mL) and the nanoparticles suspension (100 L) was added to two vials containing deionized water (4.4 mL), respectively. Deionized water (0.5 mL) was added to one vial and 35% HCl (0.5 mL) was added to the other. Thereby prepared fluorescent nanoparticles suspensions were analyzed by using the RF-500 spectrofluoro-photometer (Shimadzu, Japan). The excitation wavelength was 360 nm.

3. Results and discussion

Aiming at both of the biochip-based and the solution-based detection, our fluorescent probe (Si–Py–Ac–PEG) was designed to comprise PEGylated pyrene (Py) with acid-cleavable acetal (Ac) spacer and to be tethered to the glass/silica nanoparticle (SiNP) substrate. PEGylation provides not only the improved water solubility of hydrophobic pyrenes, but also the prevention of undesired intermolecular aggregation of pyrenes due to the increased hydrodynamic volume by the presence of conjugated PEG. The acetal groups to be hydrolyzed and transformed into aldehyde groups under acidic condition, were included as a stimuli-sensing cleavable linkage. Pyrene, a most frequently used fluorescent probe revealing its association state by excimer emission, was employed as a fluorophore. The Py–Ac–PEG was immobilized on the glass/SiNP substrate in a regular manner as to ensure that pyrenes are optimally separated for the cleavage-induced responses. As depicted in Fig. 1b, the fluorescent probes (Si–Py–Ac–PEG) are supposed to emit pyrene monomer emission under neutral aqueous condition. On the other hand, excimer emission of pyrene is expected when the probe is hydrolyzed to Si–Py structure due to the proximity and hydrophobic interaction between the Py units. Thus, Si–Py–Ac–PEG probes are expected to recognize the presence of stimuli, i.e. acidic condition in this case.

To prove the concept, Py–Ac–PEG probe was chemically conjugated on a silica glass substrate for microarray as well as on silica nanoparticles and its photophysical behavior in neutral and acidic aqueous environment was observed. The synthetic routes and chemical structures of $Si(OEt)_3$–Py–PEG (**9a**) and $Si(OEt)_3$–Py–Ac–PEG (**9b**) are depicted in Fig. 2. Bisfunctional alkylpyrene was prepared by Sonogashira coupling, followed by PEG conjugation with and without acetal acid-cleavable linkages as a sample (Py–Ac–PEG, **8b**) and a reference (Py–PEG, **8a**). Both these PEGylated pyrenes were further reacted with 3-aminopropyltriethoxysilane (APTES) to immobilize the silane compounds (**9a** and **9b**) onto the glass/SiNP. The synthesized materials were characterized with NMR, GC-Mass, MALDI-TOF Mass and elemental analysis. The UV–vis absorption and PL spectra of Py–PEG (**8a**) in neutral aqueous solution shows typical molecular pyrene behavior due to the PEGylation effect (see Fig. 3). Fig. 4 shows the results of the microarray experiments. All the images were obtained in the ArrayWorx biochip reader (Applied Precision, USA) with the excitation wavelength of 350 nm and the emission slit set to 520 nm. In neutral aqueous condition, as illustrated in Fig. 4a and c, neither Py–Ac–PEG nor Py–PEG samples displayed significant fluorescent response due to the absence of excimer emission. However, in acidic con-

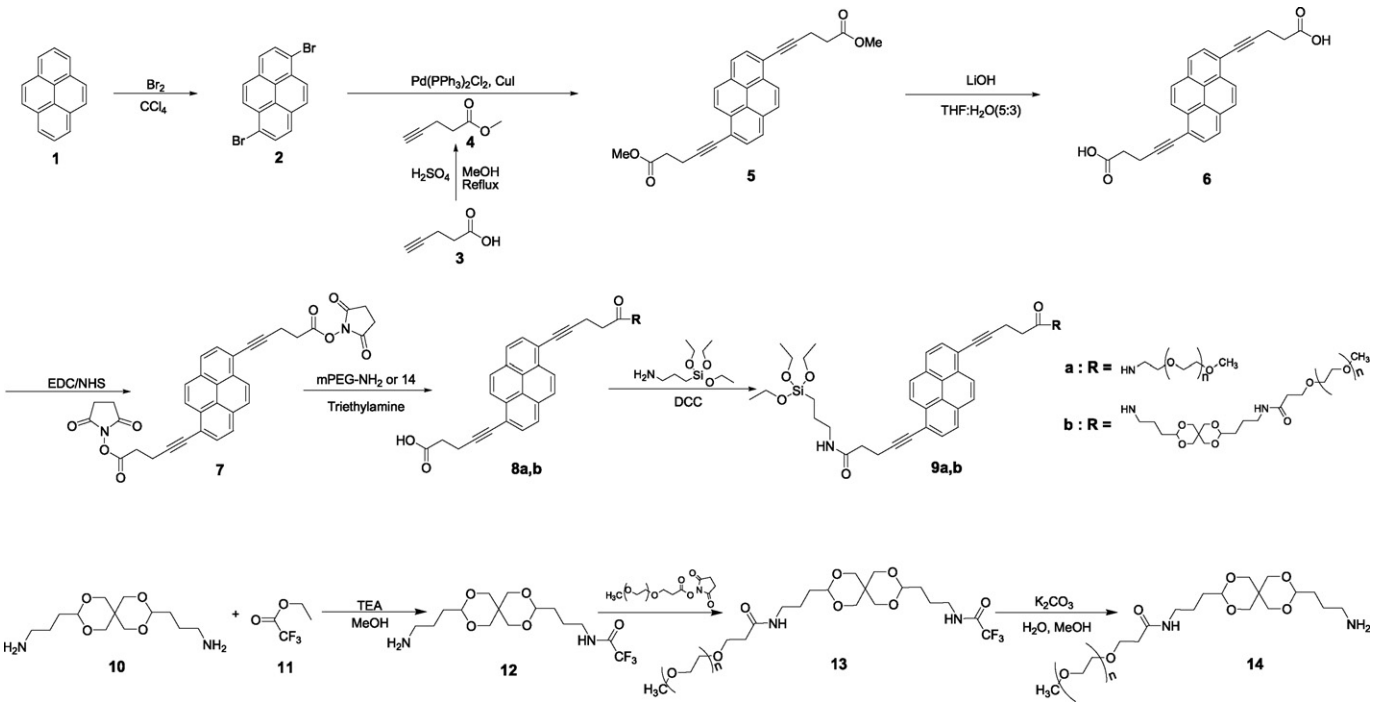

Fig. 2. Synthesis of $Si(OEt)_3$–Py–Ac–PEG (**9a**) and $Si(OEt)_3$-Py–Ac–PEG (**9b**).

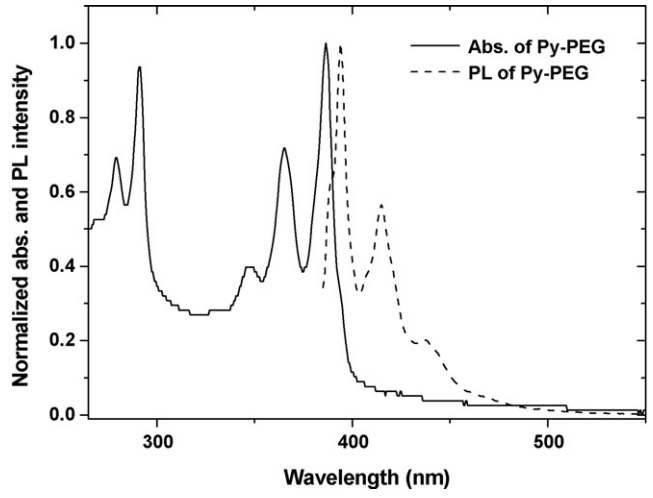

Fig. 3. Normalized UV–vis absorption and PL spectra of Py–PEG (**8a**) in water (1.7×10^{-6} M).

ditions with the addition of HCl into the arrays, fluorescence intensity was enhanced by about 6.6 times in Py–Ac–PEG array (Fig. 4b), while almost no change was effected in the case of Py–PEG array (Fig. 4d). These results from the immobilized probes on the glass substrates clearly support our hypothesis that PEGylated fluorophores with acid-cleavable linkage are capable of changing their fluorescence signals responding to the environmental conditions, the presence of acids as a stimulus in this case.

Monodisperse silica nanoparticles (SiNPs) of 150 nm diameter were prepared by the well-stabilized Stöber and Fink reaction [10]. $(EtO)_3$Si–Py–Ac–PEG (**9b**), the PEGylated acid-cleavable probes, were chemically conjugated on these SiNPs and the photoluminescent behavior in neutral and acidic aqueous solution was observed. In neutral aqueous solution, the SiNP-based probes existed in stable colloidal suspension. Upon addition of HCl into the system, however, the fluorescence of the solution was immediately changed from sky-blue to green and the aggregation of probes took place by increased hydrophobic interaction

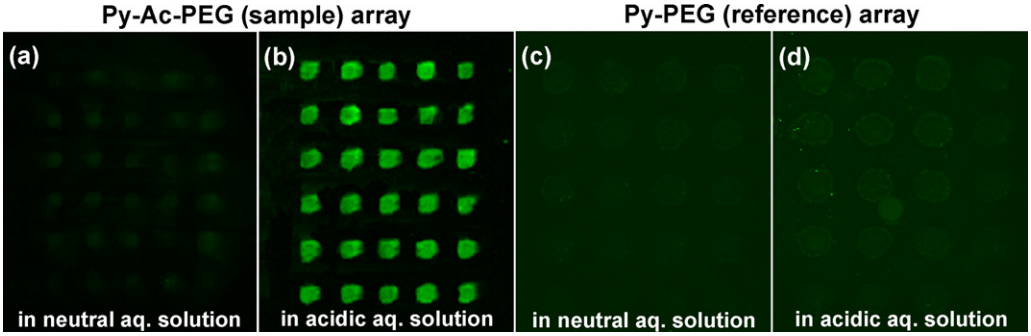

Fig. 4. Fluorescence images of spotting array of Py–Ac–PEG ((a) in water; (b) after HCl added) and Py–PEG ((c) in water; (d) after HCl added) on glass substrate. Excitation wavelength was 350 nm and emission was filtered at 520 nm.

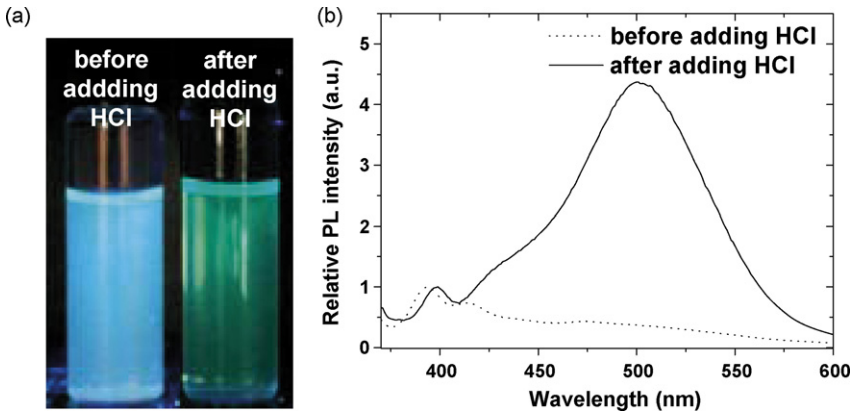

Fig. 5. (a) Fluorescence image (under 365 nm irradiation); (b) relative (emission maxima of pyrene monomer was calibrated to 1) PL spectra (excited at 360 nm) of SiNP–Py–Ac–PEG before and after HCl was added.

on the particle surfaces after the removal of hydrophilic PEG, as shown in Fig. 5a. In the steady-state emission spectrum, the intensity ratio of the excimer emission to the monomer emission (I_E/I_M) is a key photophysical parameter of excimer formation [11]. For the nanoparticle probes, the SiNP–Py–Ac–PEG emitted sky-blue fluorescence and the value of I_E/I_M was 0.4 in neutral aqueous solution. After HCl solution was added, the probe solution emitted green fluorescence with I_E/I_M value increased up to 4.3 (see Fig. 5b). These spectral data clearly confirmed the transformation from the separated monomer states of pyrenes into those of aggregated excimer after the addition of acid.

4. Conclusion

We demonstrated a novel stimuli-sensitive probe working on the principle of the cleavage-induced fluorescent change via hydrophilicity control. In our Si–Py–Ac–PEG sensor, the cleavage of hydrophilic PEG moiety in acidic environment-induced hydrophobic interaction of pyrenes to increase the excimer emission of pyrene by about 10 times. Our probe was successfully applied to the spotting microarrays and nanoparticle suspension to demonstrate the potential of biological application such as protein chip and bioimaging.

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