Journal of Fluorescence, Vol. 14, No. 5, September 2004 (©2004)

A Study of Boronic Acid Based Fluorescent Glucose Sensors

T. Kawanishi,[1,3] M. A. Romey,[1] P. C. Zhu,[1] M. Z. Holody,[1] and S. Shinkai[2]

Received November 1, 2003; accepted February 25, 2004

Boronic acid based anthracene dyes were designed, synthesized, and immobilized to solid phase, creating a continuous glucose sensor. Glucose sensitivities of dyes can decrease drastically after immobilization, therefore how to immobilize a dye to solid phase without changing the dye property is a key issue in developing the sensor. The glucose sensitivity of the simplest 1st generation sensor, which is based on an immobilized mono-phenylboronate/single-arm type, came short of the sensitivity requirement for practical use, because of the very moderate fluorescence intensity change over the physiological glucose range. However, the 2nd generation, an immobilized bis-phenylboronate/double-arm type sensor, which contained two boronate groups in the dye moiety in expectation of a large intensity change, brought about considerable improvement on its glucose sensitivity. We tried to introduce functional groups onto an anthracene ring in order to improve the dies' fluorescence properties. Acetyl or carboxyl substitution on anthracene contributed to shift the fluorescence wavelength into the more visible range (red-shift) and a divergence of wavelength between an excitation peak and an emission peak. This improvement is advantageous to the design of an optical detection system. Furthermore, single arm immobilization to this carboxyl group, thus linking directly to the fluorophore led to a 3rd generation sensor, an immobilized bis-phenylboronate/single-arm type, that was twice as sensitive as that of the 2nd generation sensor, presumably due to increased mobility of the dye moiety. The results of our study advance closer toward a clinically useful continuous fluorescent glucose sensor.

KEY WORDS: Glucose sensor; fluorescence; boronic acid; anthracene; immobilization.

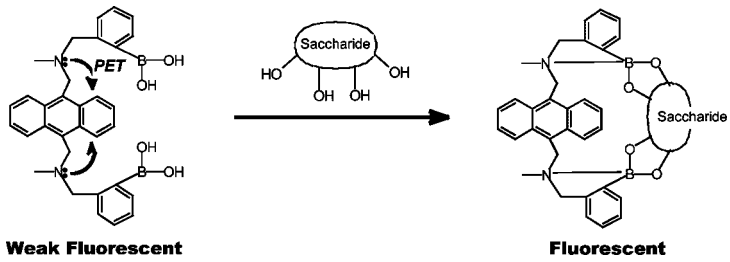
INTRODUCTION

Glucose Measurement Using Boronic Acid Based Fluorescent Dyes

There is a strong demand for glucose sensors especially in the diabetic field [1–3]. This study aims toward development of a continuous glucose monitoring system by direct blood contact, using a boronic acid based fluorescent dye. The anthracene phenylboronate compound was designed for fluorescent saccharide measurement [4–17]. The property of saccharide detection is brought out due to the mechanism shown in Scheme 1. This dye emits weak or no fluorescence in the absence of saccharide, because the fluorescence of the anthracene is quenched by the unshared electron pair of the nitrogen atom (intramolecular quenching). The electron of the nitrogen occupied the lowest excited singlet energy state of the anthracene so as to suppress the fluorescence. This phenomenon is called "photoinduced electron transfer" (PET). The presence of saccharide produces a bond between the nitrogen atom and boron atom to form a strong complex of the saccharide with these dyes, where the electron deficient boron atom has bound to the electron rich nitrogen. Thus, the unshared electron pair of the nitrogen atom will be utilized for bonding with the boron atom and will not contribute to the fluorescence-quenching process, thereby expressing the intrinsic fluorescence of the compound. Since the fluorescence intensity of the dyes is related the concentration of the existing saccharide, the concentration of saccharide can be determined by measuring the fluorescence intensity.

[1] Terumo Cardiovascular Systems Co., Tustin, California.
[2] Department of Chemistry & Biochemistry, Graduate School of Engineering, Kyushu University, Fukuoka, Japan.
[3] To whom correspondence should be addressed. E-mail: tetsuro_kawanishi@terumo.co.jp

1053-0509/04/0900-0499/0



Scheme 1. The basic mechanism of glucose sensing by fluorescent boronic acid.

Molecular Design for Immobilization

How to immobilize a dye to solid phase without any change of glucose recognition or sensitivity is a key factor for developing the dye-immobilized sensor. In order to elucidate the relationship between an immobilization form and sensor performance, three immobilization forms were designed and studied (Fig. 1). Basically, an immobilization was carried out by a reaction between the arm terminals of a dye, which are activated by introducing a reactive functional group, and an activated solid phase.

Mono-Phenyl Boronate/Single-Arm (1st Generation)

The simplest type consists of anthracene with mono-phenylboronate and single arm for immobilization, which branches off from nitrogen atom. Even though this type would be easier to synthesize, high glucose sensitivity might not be expected because the mono-phenylboronate dye itself does not have impressive glucose sensitivity compared to next-mentioned bis-phenylboronate dyes.

Bis-Phenylboronate/Double-Arm (2nd Generation)

With the intent to increase fluorescence intensity, double-arm immobilization of bis-phenylboronate dye was tried next. Both of two arms branching off from nitrogen atoms in the dye were used for immobilization. A prospective defect of this bilateral attachment may be poor mobility of the dye moiety since both glucose binding arms are tied down, thus restricting the degrees of freedom of dye movement. The restriction of dye movement could weaken interaction between dye and glucose, and might lead to loss of glucose sensitivity. Furthermore, single arm immobilization based on this design becomes more complicated since an asymmetric synthesis is needed. The synthetic process requires selective introduction of only one arm to one of two reactive secondary nitrogen atoms in a dye molecule. This asymmetric synthetic scheme was attempted but ultimately abandoned due to the difficulty in keeping the reaction limited to the production of only a single-arm product.

Bis-Phenylboronate/Single-Arm (3rd Generation)

The dye possesses a single immobilization arm attached directly to the anthracene aromatic ring. This type would solve the two disadvantages predicted in 1st and 2nd generation, i.e. bis-phenylboronate itself has good glucose sensitivity, and, also, single point attachment through the fluorophore offers greater mobility than any double-arm immobilization variation.

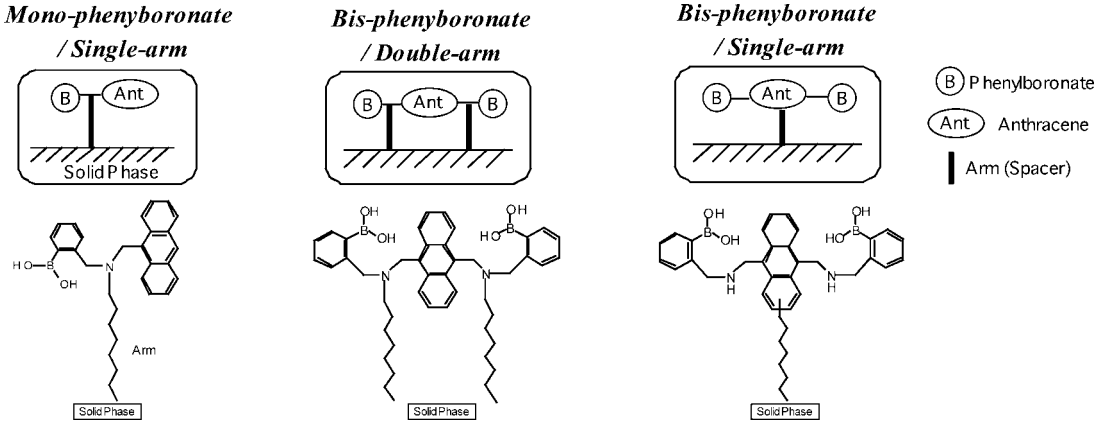


Fig. 1. Three strategies for immobilization of Shinkai dye to solid phase.

Molecular Design for Improvement of Fluorescence Properties

Non-substituted anthracene phenylboronate dyes have an excitation peak about 370 nm and an emission peak about 420 nm. These wavelengths are certainly not optimal for an optical detection system aimed at commercial products for numerous reasons. This excitation wavelength is not matched well with most commercially available blue LEDs' available peak wavelength. Furthermore, the small separation between the excitation and emission wavelengths complicates the process of discriminating between the two using filters. Therefore, it becomes important to explore new fluorophores that display longer wavelengths and larger Stokes shifts. Some researchers have examined immobilizations of mono-phenylboronate dyes. They have tried not only anthracene dyes but also dyes with different kinds of fluorophores [18,19]. However, a completely practicable sensor has not been obtained because of a loss of glucose sensitivity after immobilization. Changing the fluorophore moiety may seem a potential strategy for improving these particular fluorescence characteristics of the bis-phenylboronate type dyes. However, the selection of a fluorophore of bis-phenylboronate type is strictly limited to dye options that maintain the proper distance between both boronic acid moieties so as to permit adequate glucose binding and recognition. With the consideration of the circumstance, we studied improvement of the fluorescence characteristics by introduction of a functional group onto the anthracene molecule. Theoretically substitution of aromatic rings by electron-attracting functional groups may permit red-shift in the dye's excitation and emission spectra. To this end, introduction of acetyl group into anthracene ring of bis-phenylboronate dye was studied.

Immobilization Protocol

A regenerated cellulose membrane is used as a solid phase, because it (1) is easy to activate or modify its surface, (2) has no absorbance and is not fluorescent, and (3) has a good biocompatibility. When thinking of application of such a glucose sensor, one concern was the possibility of a glucose unit of cellulose interacting with boronic acid of a dye. This undesirable interaction might cause interference of glucose detection, or nonspecific dye binding during the immobilization reaction. Nonspecific binding between cellulose and a dye molecule was observed in several conditions of the immobilization reaction. However, glucose detection was not affected. Technologies of immobilization reactions have been developed in the realm of affinity chromatography and solid phase organic synthesis [20–23]. Some established methods for attachment of antibodies, nucleic acids, active proteins etc. to solid phase, can be applied to our sensor chemistry.

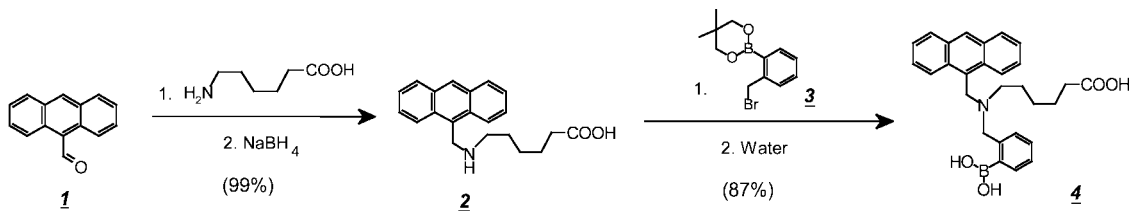
EXPERIMENTAL

Anthracene Mono-Phenylboronate/Single-Arm

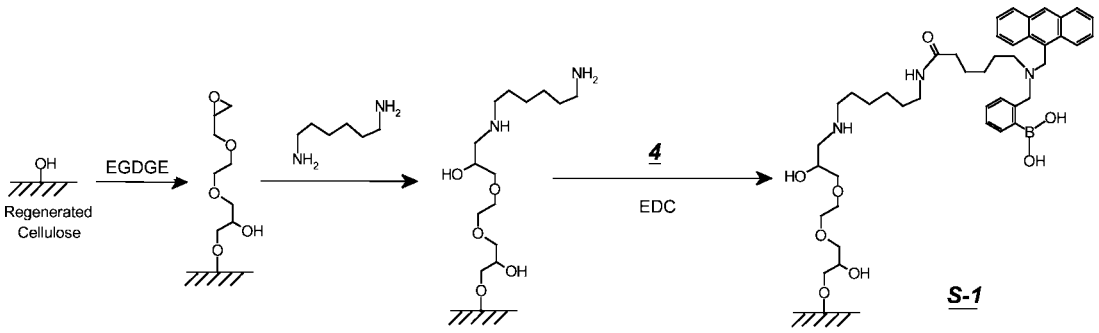
Synthesis (Scheme 2)

Synthesis of ***2***. 6-Aminocaproic acid (5.0 g, 38.1 mmol) reacted with 9-anthracene-carboxaldehyde (**1**) (3.93 g, 19.1 mmol) in 750 mL methanol in the presence of potassium carbonate at room temperature for 1 hr. After reduction by sodiumborohydride (3.61 g, 95.3 mmol), secondary amine (**2**) was given: yield 6.06 g (99 %): ^{1}H NMR (DMSO-d_6)δ 1.35–1.70 (m, 6H, C—CH_2—C), 2.50 (t, 2H, CH_2—CO), 3.20 (t, 2H, N—CH_2—C), 5.20 (s, 2H, N—CH_2—anthracene), 7.60–8.80 (m, 9H, anthracene).

Synthesis of ***4***. 2,2-Dimethylpropane-1,3-diyl[*o*-(bromomethyl)phenyl]borate (***3***) was synthesized in the established manner [8]. The mixture of ***3*** (2.83 g, 10.0 mmol), **2** (3.21 g, 10.0 mmol), sodium carbonate (2.12 g, 20.0 mmol), and 216 mL of acetonitrile was refluxed for 23 hr. The required carboxylic acid (***4***) was obtained following cleavage of the boronate protecting group by treating with 40 mL of water: yield 3.96 g (87 %); ^{1}H NMR (DMSO-d_6) δ 0.90–1.70 (m, 6H, C—CH_2—C), 2.40 (t, 2H, CH_2—COO), 3.15 (t, 2H, N—CH_2—C), 3.85 (m, 2H, N—CH_2—benzene), 4.45 (s, 2H, N—CH_2—anthracene), 7.10–8.65 (m, 13H, aromatic).



Scheme 2. Synthesis of anthracene mono-phenylboronate/single-arm carboxylic acid terminated (***4***).



Scheme 3. Immobilization of anthracene mono-phenylboronate/single-arm (**4**) to regenerated cellulose membrane.

Immobilization (Scheme 3)

EGDGE Activation. Ethylene glycol diglycidyl ether (EGDGE) (625 mg, 3.59 mmol) was dissolved in 2 mL of dimethylsulfoxide (DMSO). 60 μL of 50% aqueous sodium hydroxide and 8 mL of water was added to the solution. Eight pieces of 1.8 cm $\times$ 1.8 cm size of square regenerated cellulose membrane (Cuprophan®) were soaked in the solution gently. After reacting at room temperature for 20 min, the solution was removed. The sheets were washed in 20 mL of water four times.

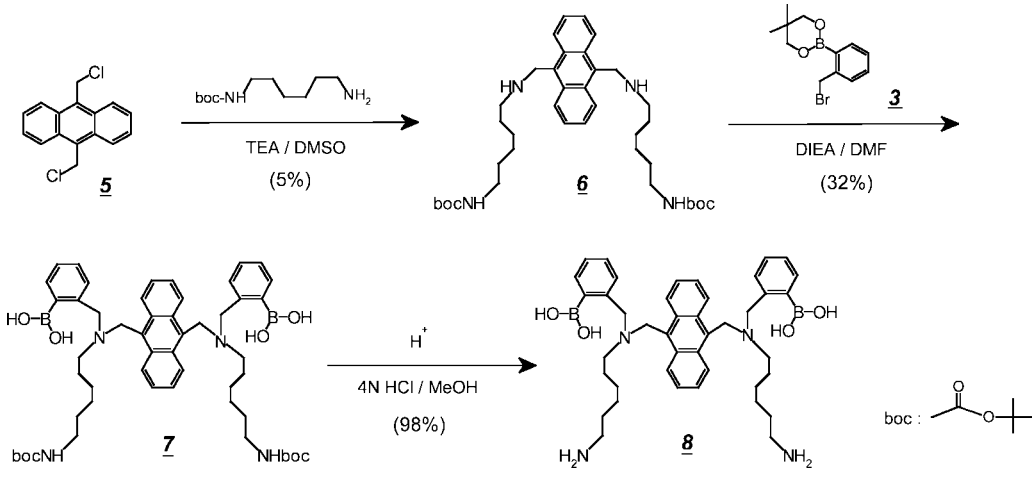
HDA Activation. Four pieces of EGDGE activated membrane were soaked in 10 mL of 4.2% (w/v) 1,6-hexanediamine (HDA) aqueous solution and reacted for another 2 hr. The solution was removed by a pipette, and the sheets were washed with water five times and tetrahydrofuran (THF) twice.

*Immobilization of **4*** (**S-1**). The mixture of **4** (3 mg, 4.27 μmol), 1-[3-(dimetylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (6 mg, 31.3 μmol), 1-hydroxybenzotriazol hydrate (HOBt) (4.0 mg, 30 μmol), and DIEA (25 μL, 0.14 mmol) were dissolved in 1.0 mL of dimethylformamide (DMF). Two pieces of glutaric acid activated regenerated cellulose membrane were soaked in the solution. After reacting at room temperature for 16 hr, the dye solution was removed. The sheets were washed by DMF twice, 0.01 N hydrochloric acid twice, and water three times. Fluorescent membranes were obtained.

Anthracene Bis-Phenylboronate/Double-Arm

Synthesis (Scheme 4)

*Synthesis of **6***. Bis(chloromethyl)anthracene (500 mg, 1.82 mmol), N-BOC-1,6-diaminohexane (1.6 g, 6.33 mmol), and triethylamine (TEA) (1.76 mL, 12.6 mmol) were dissolved in 10 mL of anhydrous DMSO. The mixture was stirred at 55°C for 4 hr. The mixture was pour into 60 mL of ice-cold water slowly. The solid was collected on a Buchner funnel and allowed to dry under reduced pressure. The solid was dissolved in 2 mL of



Scheme 4. Synthesis of anthracene bis-phenylboronate/double-arm (**8**).

chloroform and applied to silica gel column chromatography eluted with methanol/chloroform. The fluorescent fraction, which appears at Rf 0.15 on TLC (silica gel, 12% methanol in chloroform for developer, detected by 254 nm UV light), was collected, evaporated, and then dried under reduced pressure: yield 32.6 mg (5%); ^{1}H NMR ($CDCl_3$) δ 1.30–1.75 (m, 16H, C—CH2—C), 1.47 (s, 18H, t-Bu), 2.80 (t, 4H, N—CH2—C), 3.15 (m, 4H, CH2—N-boc), 4.74 (s, 4H, N—CH2—anthracene), 8.00 (m, 8H, anthracene).

Synthesis of **7**. **6** (26 mg, 0.0472 mmol), **3** (40 mg, 0.142 mmol), and DIEA (10 μL, 0.057 mmol) were dissolved in 1.0 mL of DMF. The mixture was stirred at room temperature for 5 hr. The reaction mixture was diluted with 20 mL of chloroform, washed with water twice, washed with saturated aqueous sodium chloride, and dried over anhydrous sodium sulfate. After removal of sodium sulfate by filtration, the filtrate was evaporated, and applied to silica gel column chromatograph eluted with methanol/chloroform. The fluorescent fraction, which appears at Rf 0.6 on TLC (silica gel, 12% methanol in chloroform for developer, detected by 254 nm UV light), was collected, evaporated, and then dried under reduced pressure: yield 14.0 mg (32%); ^{1}H NMR ($CDCl_3$); δ 1.00–1.60 (m, 16H, C—CH_2—C), 1.47 (s, 18H, t-Bu), 2.58 (m, 4H, N—CH_2—C), 2.85 (m, 4H, CH_2—N-boc), 3.95 (s, 4H, N—CH_2—benzene), 4.50 (s, 4H, N—CH_2—anthracene), 7.42 and 8.10 (m, 16H, aromatic).

Synthesis of ***8***. **7** (10 mg, 0.011 mmol) was added to the mixture of 0.5 mL of methanol and 0.4 mL of 4 N hydrochloric acid. The solution was stirred at 50°C for 30 min. The progress of the reaction was checked by TLC (silica gel, chloroform/methanol/ammonia (70/27/3) for developer, detected by 254 nm UV light). The Rf-values of required product was 0.2. The spots of unprotected products on TLC were colored by after spraying ninhydrin solution. The reaction mixture was evaporated, dried under reduced pressure. 10 mg of **8** including small amount of inorganic salt was obtained quantitavely. The product can be used for assays or immobilizations without further purification: ^{1}H NMR (DMSO-d_6) δ 1.00–1.70 (m, 16H, C—CH_2—C), 2.75 (m, 4H, N—CH_2—C), 2.90 (m, 4H, CH_2—NH_2), 3.85 (s, 4H, N—CH_2—benzene), 4.60 (s, 4H, N—CH_2—anthracene), 7.45–8.25 (m, 16H, aromatic).

Immobilization (Scheme 5).

To attach anthracene bis-phenylboronate/double-arm dye (**8**) to cellulose membrane, three immobilization methods were tried as shown in Scheme 5. Surface analysis by titration seems to indicate that both arms of each dye are occupied.

Glutaric Acid Activation. The mixture of glutaric anhydride (1.0 g, 8.76 mmol) and 4-dimetylaminopyridine (DMAP) (10 mg, 0.082 mmol) was dissolved in the mixture of 2 mL of THF and 0.4 mL (2.29 mmol) of diisopropylethylamine (DIEA). Two pieces of HDA activated membranes were soaked in the dye solution. After reacting at room temperature for 19 hr, the solution was removed. The sheets were washed with THF three times, water twice, sodium bicarbonate buffer twice, water twice again, and DMF twice.

Immobilization of ***8*** *to Glutaric Acid Activated Membrane* (**S-2**). The mixture of **8** (3 mg, 4.27 μmol), EDC (6 mg, 31.3 μmol), HOBt (4.0 mg, 30 μmol), and DIEA (25 μl, 0.14 mmol) were dissolved in 1 mL of DMF. Two pieces of aforementioned glutaric acid activated regenerated cellulose membrane were soaked in the solution. After reacting at room temperature for 16 hr, the dye solution was removed. The sheets were washed by DMF twice, 0.01N hydrochloric acid twice, and water three times.

Cyanogenbromide (CNBr) Activation. Two of 1.8 cm $\times$ 1.8 cm sizes of regenerated cellulose membrane were gently soaked in the mixture of 100 μL of freshly prepared 10 mg/mL cyanogenbromide aqueous solution and 2.0 mL of 1 M K_3PO_4 aqueous solution. After reacting at room temperature for 10 min, the solution was removed. The sheets were washed with water three times, DMF three times.

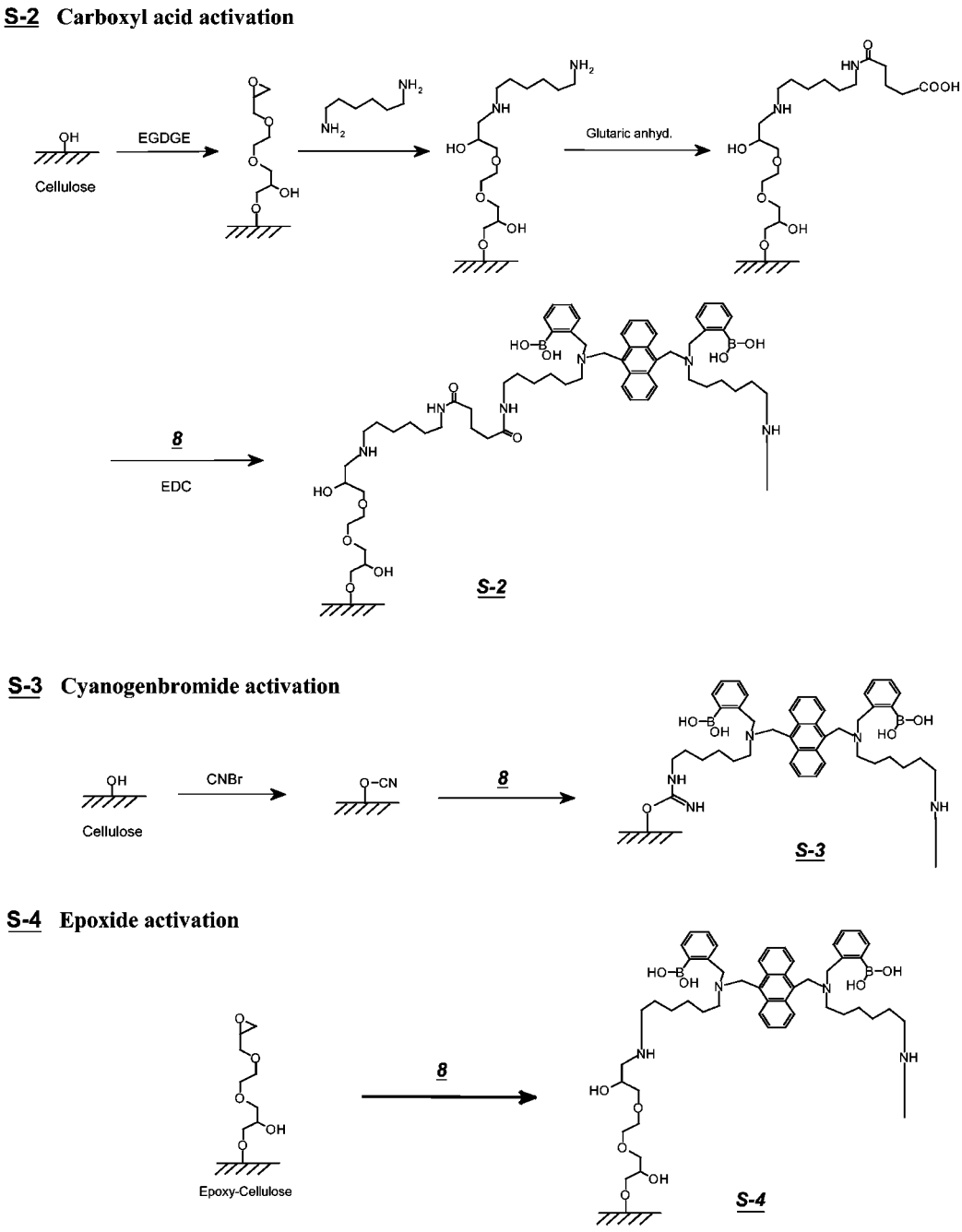
Immobilization of ***8*** *to CNBr Activated Membrane* (S-3). Two pieces of CNBr activated membrane were gently soaked in the mixture of **8** (4.6 mg, 6.55 μmol), 0.5 mL of DMF, and 40 μL of DIEA. After reacting at room temperature for 3 hr, the dye solution was removed. The sheets was washed with DMF three times, water three times, and 50 mM phosphate buffer (pH = 7.0) three times.

Immobilization of ***8*** *to EGDGE Activated Membrane* (**S-4**). Two pieces of foresaid EGDGE activated cellulose membrane were soaked in the mixture of **8** (4.6 mg, 6.55 μmol), 40 μL (0.23 mmol) of DIEA, and 0.5 mL of DMF. Two sheets of EGDGE activated membrane were soaked in the dye solution. After reacting at room temperature for 5.5 hr, the dye solution was removed. The sheets was washed with DMF three times, water three times, 100 mM sodiumbicarbonate buffer (pH = 7.0) twice, 2 M sodiumchloride twice, and water twice again.

Acetylanthracene Bis-Phenylboronate/Double-Arm

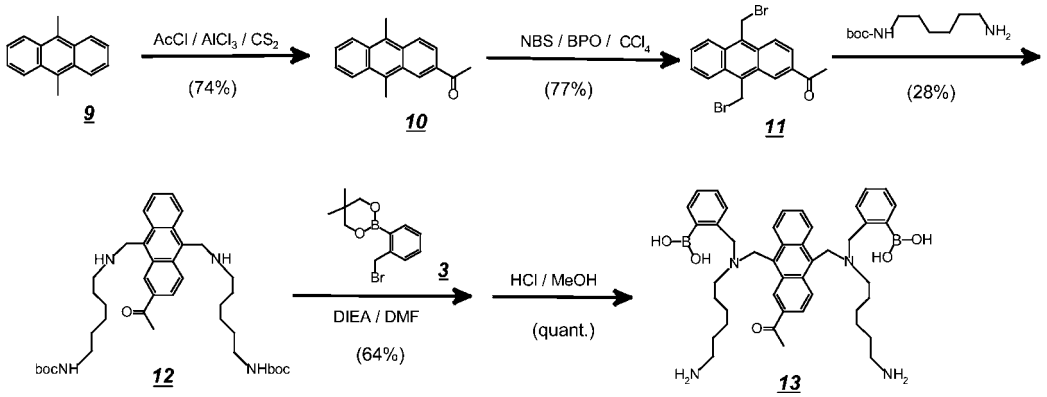
Synthesis (Scheme 6)

Synthesis of ***10***. Aluminum chloride (870 mg, 6.52 mmol) was added to the mixture of 9,10-dimethylanthracene (**9**) (930 mg, 4.50 mmol), distilled



Scheme 5. Three different immobilization forms of anthracene bis-phenylboronate/double-arm (**8**) to regenerated cellulose membrane.

acetylchrolide (0.5 mL, 7.03 mmol) and 40 mL of carbondisulfide. After stirring at room temperature overnight, the mixture allowed to heat on an oil bath at 45C for 2 hr with stirring. 15 mL of cracked ice with 0.8 mL hydrogen chloride was added. The mixture was extracted with 40 mL of chloroform, washed with water, and dried over anhydrous sodium sulfate. The chloroform solution was evaporated, and purified by silica gel column chromatograph eluted with chloroform: yield 825 mg (74 %); ^{1}H NMR ($CDCl_3$)δ 2.80 (s, 3H, Ac), 3.15 (d, 6H, CH_3—anthracene), 7.60–9.00 (m, 7H, anthracene).



Scheme 6. Synthesis of acetylanthracene bis-phenylboronate/double-arm (**13**).

*Synthesis of **11***. A mixture of 2-acetyl-9,10-dimethylanthracene (**10**) (340 mg, 1.37 mmol), of *N*-bromosuccinimide (540 mg, 3.03 mmol), benzoylperoxide (5.0 mg, 20.6 μmol), 4 mL of chloroform, and 10 mL of carbontetrachloride was refluxed for 1.8 hr. The solvent was removed completely by evaporation, and 10 mL of methanol was added. The mixture was stirred for 10 min, and the solid was filtered and washed with 2 mL of methanol. The solid was dried under reduced pressure: yield 430 mg (77%); ^{1}H NMR ($CDCl_3$) δ 2.80 (s, 3H, Ac), 5.50 (d, 4H, Br—CH_2—anthracene), 7.50–9.00 (m, 7H, anthracene).

*Synthesis of **12***. A mixture of 2-acetyl-9,10-bis (boromomethyl)anthracene (430 mg, 1.06 mmol), N-BOC-1,6-diaminohexane (1.07 g, 4.23 mmol), 6.0 mL of DMF, and 1.0 mL (5.74 mmol) of DIEA was stirred at 45C for 1 hr. The mixture was diluted with 30 mL of chloroform, washed with water twice, and washed with saturated aqueous sodium chloride, and dried. The solution was evaporated, and applied to silica gel column chromatograph eluted with methanol/chloroform: yield 219 mg (28%); ^{1}H NMR ($CDCl_3$) δ 1.25–1.70 (m, 16H, C—CH_2—C), 1.45 (s, 18H, t-Bu), 2.80 (s, 3H, Ac), 2.90 (m, 4H, N—CH_2—C), 3.15 (m, 4H, CH_2—N-boc), 3.95 (m, 4H, N—CH_2—benzene), 4.75 (d, 4H, N—CH_2—anthracene), 7.20–9.30 (m, 15H, aromatic).

The synthesis of **12** is almost the same reaction as the **5**–**6** step. The only difference between them is whether the acetyl group exists on the anthracene ring. However the yield of **12** (28%) is better than that of **6** (5%). This improvement would be due to solvent effects. In the **5**–**6** step, DMSO is the only solvent of choice, because only DMSO dissolves the starting material. On the other hand, the acetylated starting material for the **11**–**12** step was soluble in dimethylformamide (DMF). It seems that DMF was the more suitable solvent for this reaction, judging from the improved yield with its use.

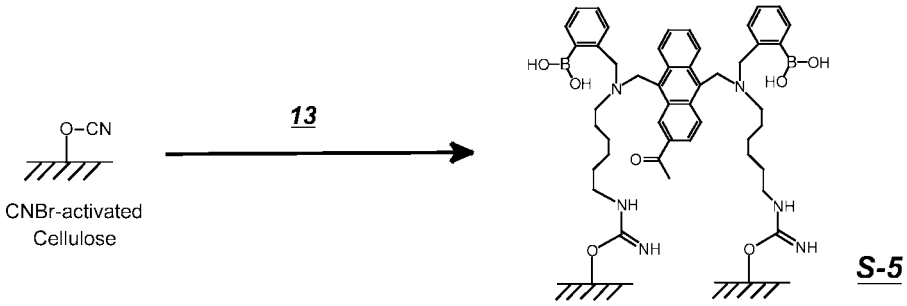
*Synthesis of **13***. A mixture of **11** (219 mg, 0.292 mmol), **3** (330 mg, 1.172 mmol), 4.5 mL of DMF, and 0.15 mL (0.865 mmol) of DIEA was stirred at 40C for 1 hr. The mixture was diluted with 30 mL of chloroform, washed with water twice, and washed with saturated aqueous sodium chloride, and dried. The solution was evaporated, and applied to silica gel column chromatograph eluted with methanol/chloroform: yield 192 mg (64%). The product (192 mg, 0.188 mmol) was dissolved in 5 mL of methanol, and 4 N hydrochloric acid was added. After the mixture was stirred at room temperature for 18 hr, evaporated and dried. 188 mg of yellow powder was obtained as the final product: ^{1}H NMR (DMSO—d_6) δ 1.20–1.80 (m, 16H, C—CH_2—C), 2.75 (s, 3H, Ac), 2.90 (m, 4H, N—CH_2—C), 3.00 (m, 4H, CH_2—NH_2), 4.10 (m, 4H, N—CH_2—benzene), 4.80 (d, 4H, N—CH_2—anthracene), 7.10–9.00 (m, 15H, aromatic). The product contains a little inorganic salt. However, it was used for assay or immobilization without further purification.

Immobilization (**S-5**, Scheme 7)

Two pieces of CNBr activated membrane were gently soaked in the mixture of **13** (5.0 mg, 6.72 μmol), 0.49 mL of DMF, and 10 μL (0.057 mmol) of DIEA. After reacting at 5 C for 17 hr, the dye solution was removed. The sheets was washed with DMF three times, water three times, and soaked in 50 mM phosphate buffer (pH = 7.0) at 5°C for 5 days before use.

Anthracene Bis-Phenylboronate/Single-Arm

Assuming that the loss of sensitivity after immobilization is related to the reduced dye mobility, we considered immobilization schemes that could allow the immobilized dye to have more degrees of freedom. We tried single-arm immobilization direct from the anthracene ring. A carboxyl group on anthracene, which



Scheme 7. Immobilization of acetylanthracene bis-phenylboronate/double-arm (**13**).

worked as an active functional group for immobilization, was derived from the acetylathracene intermediate synthesized in the preceding chapter.

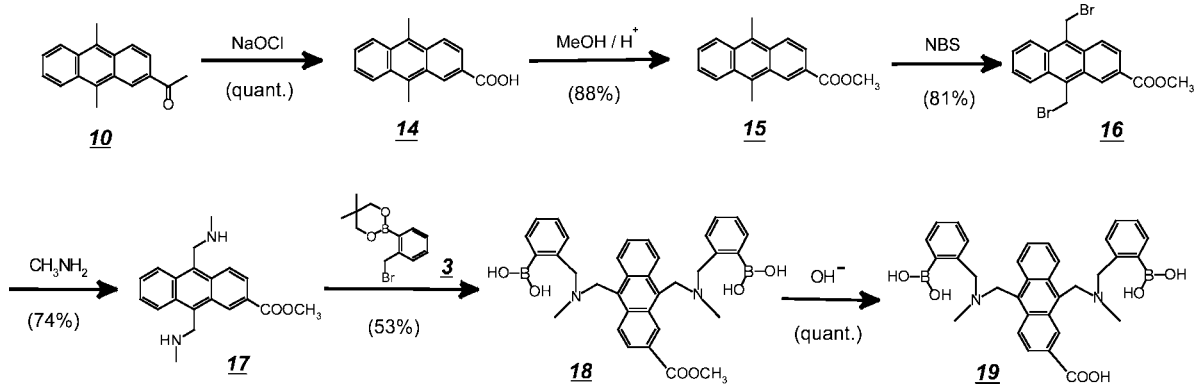
Synthesis (Scheme 8)

*Synthesis of **14***. 2-Acetyl-9,10-dimethylanthracene (**10**) (536 mg, 2.16 mmol) was dissolved in 12.5 mL of dioxane in a flask. A mixture of 5.0 mL of 10–13% sodium hypochlorite and 3.5 mL of 6.7% (w/v) sodium hydroxide aqueous solution was added to the flask with stirring. The mixture was stirred at 85°C for 8 hr. After cooling to room temperature, 5 mL of water was added, and the mixture was acidified with hydrochloric acid. Formed precipitate was filtered, washed with small amount of water, and dried under reduced pressure overnight. 579 mg of yellow powder containing a little inorganic salt was obtained. It was used for the next step without further purifications: ^{1}H NMR (DMSO-d_6) δ 3.15 (d, 6H, CH_3), 7.60–9.00 (m, 7H, anthracene).

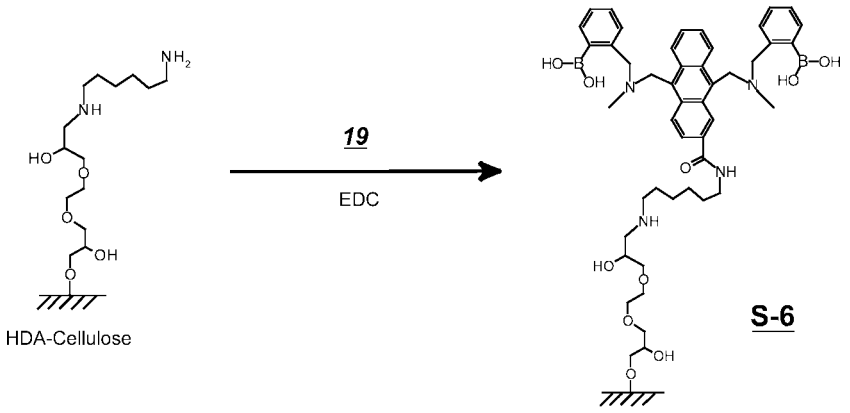
*Synthesis of **15***. A mixture of **14** (150 mg, 0.60 mmol), 80 mL of absolute methanol, and 0.2 mL of sulfuric acid was refluxed for 20 hr. The mixture was concentrated to about 10 mL by evaporation, and diluted with 50 mL of chloroform. The mixture was washed with 5% sodium bicarbonate aqueous solution twice, saturated aqueous sodium chloride, and dried: yield 140 mg (88%); ^{1}H NMR ($CDCl_3$) δ 3.15 (d, 6H, CH3-ϕ), 4.00 (d, 3H, $COOCH_3$), 7.55–9.20 (m, 7H, anthracene).

*Synthesis of **16***. A mixture of 2-acetyl-9,10-dimethylanthracene (**10**) (360 mg, 1.36 mmol), of *N*-bromosuccinimide (540 mg, 3.03 mmol), benzoylperoxide (5.0 mg, 20.6 μmol), 4 mL of chloroform, and 10 mL of carbontetrachloride was refluxed for 1.8 hr. The solvent was removed completely by evaporation, and 10 mL of methanol was added. The mixture was stirred for 10 min, and the solid was filtered and washed with 2 mL of methanol. The solid was dried under reduced pressure: yield 430 mg (77%); ^{1}H NMR ($CDCl_3$) δ 4.00 (s, 3H, $COOCH_3$), 5.80 (d, 4H, Br—CH_2-ϕ), 7.55–9.20 (m, 7H, anthracene).

*Synthesis of **17***. A mixture of **16** (50 mg, 0.118 mmol), 1.0 mL (2 mmol) of 2 M methylamine methanol solution, and 8 mL of chloroform was stirred at room temperature for 4 hr. Suspended reaction solution becomes clear in first 1 hr. The solvent was removed completely by evaporation, and residue was purified by silica gel column chromatograph eluted with methanol/chloroform: yield 28 mg (74%); ^{1}H NMR (DMSO—d_6) δ 2.80 (d, 6H, N—CH_3), 4.00 (s, 3H, $COOCH_3$), 5.25 (m, 4H, N—CH_2-anthracene), 7.80–9.20 (m, 7H, anthracene).



Scheme 8. Synthesis of carboxylanthracene bis-phenylboronate (**19**).



Scheme 9. Immobilization of anthracene bis-phenylboronate carboxylic acid (**19**) to cellulose membrane.

*Synthesis of **18***. **17** (200 mg, 0.62 mmol), **3** (790 mg, 2.81 mmol), 3.0 mL of DMF, and 0.35 mL (2.01 mmol) of DIEA was stirred at room temperature 16 hr. The reaction mixture was diluted with 40 mL of chloroform, washed with water twice, washed with of saturated aqueous sodium chloride, and dried. The solution was evaporated, and applied to silica gel column chromatograph eluted with methanol/chloroform: yield 194 mg (53 %); ^{1}H NMR ($CDCl_3$) δ 2.20 (d, 6H, N—CH_3), 3.94 (m, 4H, N—CH_2—benzene), 4.02 (s, 3H, $COOCH_3$), 4.52 (m, 4H, N—CH_2—anthracene), 7.45–9.05 (m, 15H, aromatic).

*Synthesis of **19***. **18** (154 mg, 0.26 mmol) was dissolved in 4 mL of methanol, and 1.5 mL of 3 N sodium hydroxide aqueous solution was added. The mixture was stirred at 70°C for 5 hr. The mixture was treated with prewashed cation exchange resin to remove alkaline. The solution was evaporated and dried. 188 mg of yellow powder was obtained as the final product: mp = 121 C, ^{1}H NMR (DMSO-d_6) δ 2.15 (d, 6H, N—CH_3), 4.10 (m, 4H, N—CH_2—benzene), 4.45 (m, 4H, N—CH_2—anthracene), 7.55–8.90 (m, 15H, aromatic). The product contains a little inorganic salt. However, it was used for assay or immobilization without further purification.

Immobilization (**S-6**, Scheme 9)

19 (5.0 mg, 8.68 μmol), EDC (6.0 mg, 31.3 μmol), HOBt (4.0 mg, 30 μmol) were dissolved in 1.0 mL of DMF. Two pieces of HDA activated regenerated cellulose membrane were soaked in the solution. After reacting at room temperature for 17 hr, the dye solution was removed. The sheets were washed by DMF three times, water for 20 min each three times, and soaked in 50 mM phosphate buffer (pH = 7.0) for 1 hr before use.

Fluorescence Measurements

A spectrofluorometer, SLM-Aminco 4800C (Urbana, IL), was used for all fluorescence measurements. For solution assays, sample solution was transferred in quartz cell (3 mL, 1 cm light-path length) and excitation or emission spectra were recorded. Common setting of the instrument is follows: slit = 4–4–4, scan resolution = 1 nm, gain and high voltage (HV) = described in each data. For assays on sensor membranes, a membrane was cut out to 6 × 6 mm square size and fixed into a sensor cassette specially designed for a series of tests. Buffer solution can be circulated inside the cassette that was equipped on an optical head and contacted a surface of a sensor membrane. The optical head consists of a cassette fitted with optical contact ports containing a batch of optical fibers. Half portions of the optical fibers carry the excitation light from the spectrofluorometer to the test membrane sample, and the other half carry the emitted light back to the photo detector to measure a fluorescence emission.

RESULTS AND DISCUSSION

Fluorescence Property of the Synthesized Dyes and the Obtained Sensor Membranes

*Fluorescence Properties of Bis- and Mono-Phenylboronate Dyes in Solution (**4** & **8**)*

Both mono-phenylboronate/single-arm dye (**4**) and bis-phenylboronate/double-arm dye (**8**) gave similar fluorescence spectra in buffer solution as shown in Fig. 2. These spectra were particular to anthracene-based dyes, thus it appears that the introduction of arms into an amino group has no influence on fluorescence spectrum

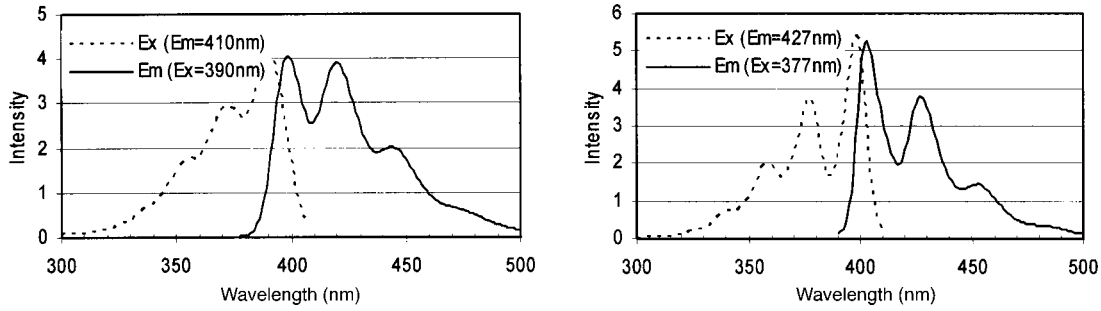


Fig. 2. Fluorescence spectra of anthracene mono-phenylboronate/single-arm (**4**, left) and anthracene bis-phenylboronate/double-arm (**8**, right) in 33% MeOH/PBS; dye concentration: 0.01 mM, instrument: SLM4800 (HV = 750, Gain = X10).

properties of the dye. After immobilization of **4** and **8**, the characteristics of spectra have not changed much. (the spectra of **S-1** to **S-4** are not shown)

*Fluorescence Property of Acetylanthracene Dye in Solution (**13**)*

Figure 3 compares fluorescence spectra of bis-phenylboronate dyes both with (**13**) and without acetyl group (**8**). Introduction of acetyl group caused the dye to exhibit both a red-shift and a Stokes' shift. Excitation spectra of both are similar, but the emission peak of the acetyl dye shifted to a longer wavelength. The acetyl dye has about 480 nm emission peak, whereas the non-substitute compound has 405 nm and 428 nm emission peaks. The result clearly indicates that introducing an acetyl group onto an anthracene ring contributes to shifting the emission peak wavelength toward longer wavelength region.

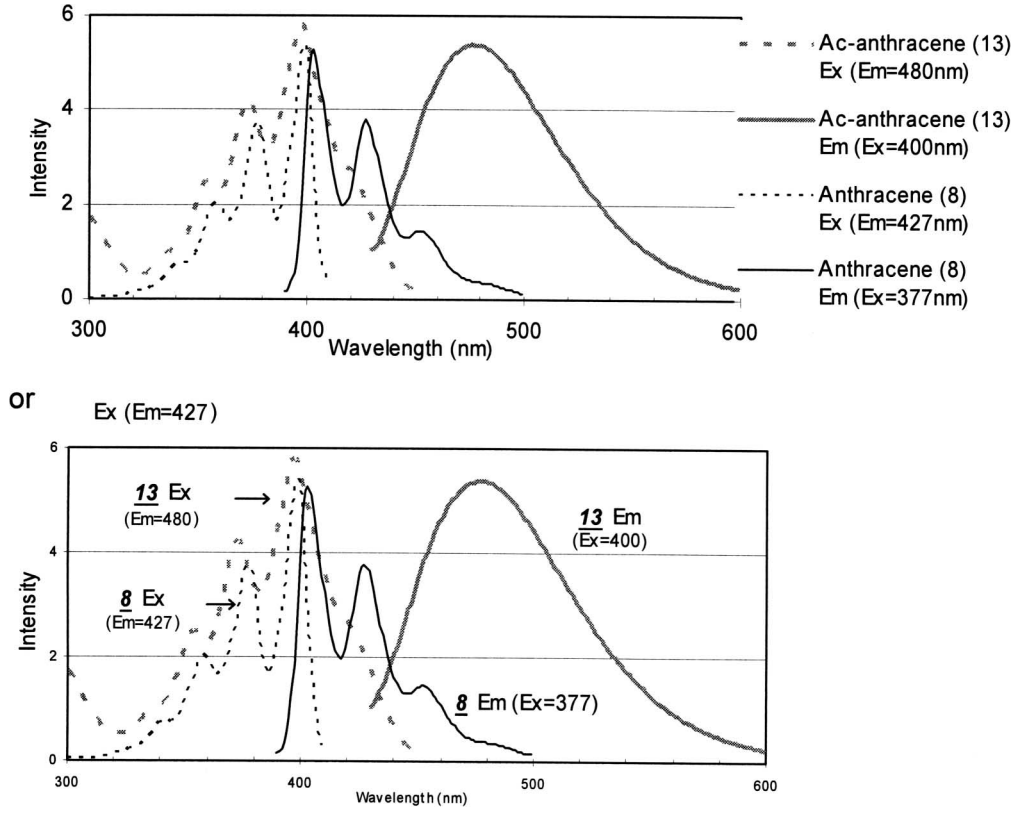
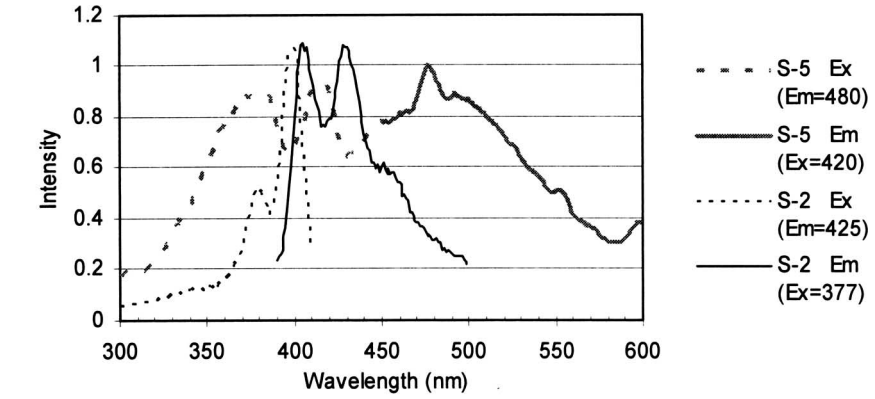


Fig. 3. Fluorescence spectra of acetylanthracene bis-phenylboronate/double-arm (**13**) and anthracene bis-phenylboronate/double-arm (**8**) in 33% MeOH/PBS; dye concentration: 0.01 mM, instrument: SLM4800 (HV = 750, Gain = X10).



or

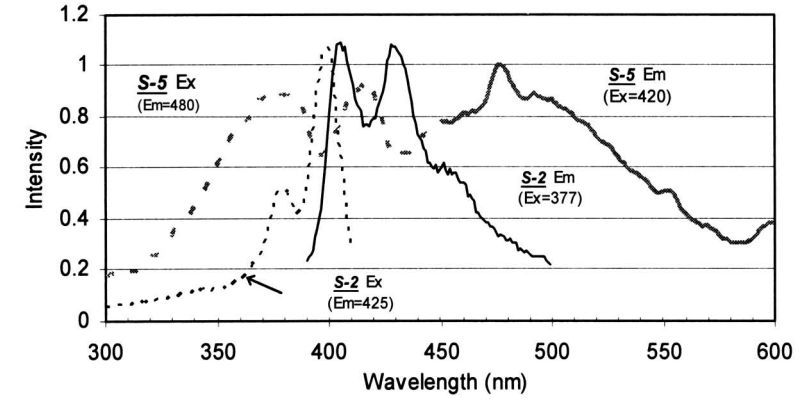


Fig. 4. Fluorescence spectra of the sensor membrane, which consists of acetylanthracene bis-phenylboronate/double-arm immobilized to cellulose membrane (**S-5**), and anthracene bis-phenylboronate/double-arm immobilized to cellulose membrane (**S-2**), in PBS (pH = 7.4), instrument: SLM4800 (HV = 850, Gain = X100).

Fluorescence Property of Acetylanthracene Sensor Membrane **(S-5)**

After immobilization of **13**, it still kept a good fluorescence characteristic in its spectrum. Fig. 4 shows that this sensor (**S-5**) has a much-improved fluorescence property compared to non-substituted anthracene derivative (**S-2**). This improvement helps avoid the need to build a complicated optical detection system.

*Fluorescence Property of Carboxylic Acid Dyes in Solution (**18 & 19**)*

Fig. 5 shows the comparison of fluorescence spectra among carboxylic dyes that were developed for single arm immobilization (**18**), ester of carboxylic acid (**19**), and non-substituted dye (**8**) in buffer solution. Excitation spectra were similar to each other, however, different emission characteristics were observed. The carboxylic acid (**19**) forms a red-shifted emission curve as compared to non-substituted dye (**8**), even though emission peak wavelength is lower than that of the acetyl dye (**13**). The emission peak of the methyl ester (**18**) lies in a little longer wavelength region than that of the carboxylic acid. It is concluded that the carboxylic dye still keeps red-shifted behavior, but the degree is smaller than that of acetyl dye. The immobilized carboxylic acid (**S-5**) still keeps the favorable fluorescence property, in which excitation peak wavelength is 405 nm and emission peak is 450 nm. (the spectrum of **S-5** is not shown)

Glucose Sensing Performances of Dyes in Buffer Solution

Glucose response curves of synthesized dyes in buffer solutions are shown in Fig. 6. Anthracene monophenylboronate type (**4**) gave a poor sensitivity. On

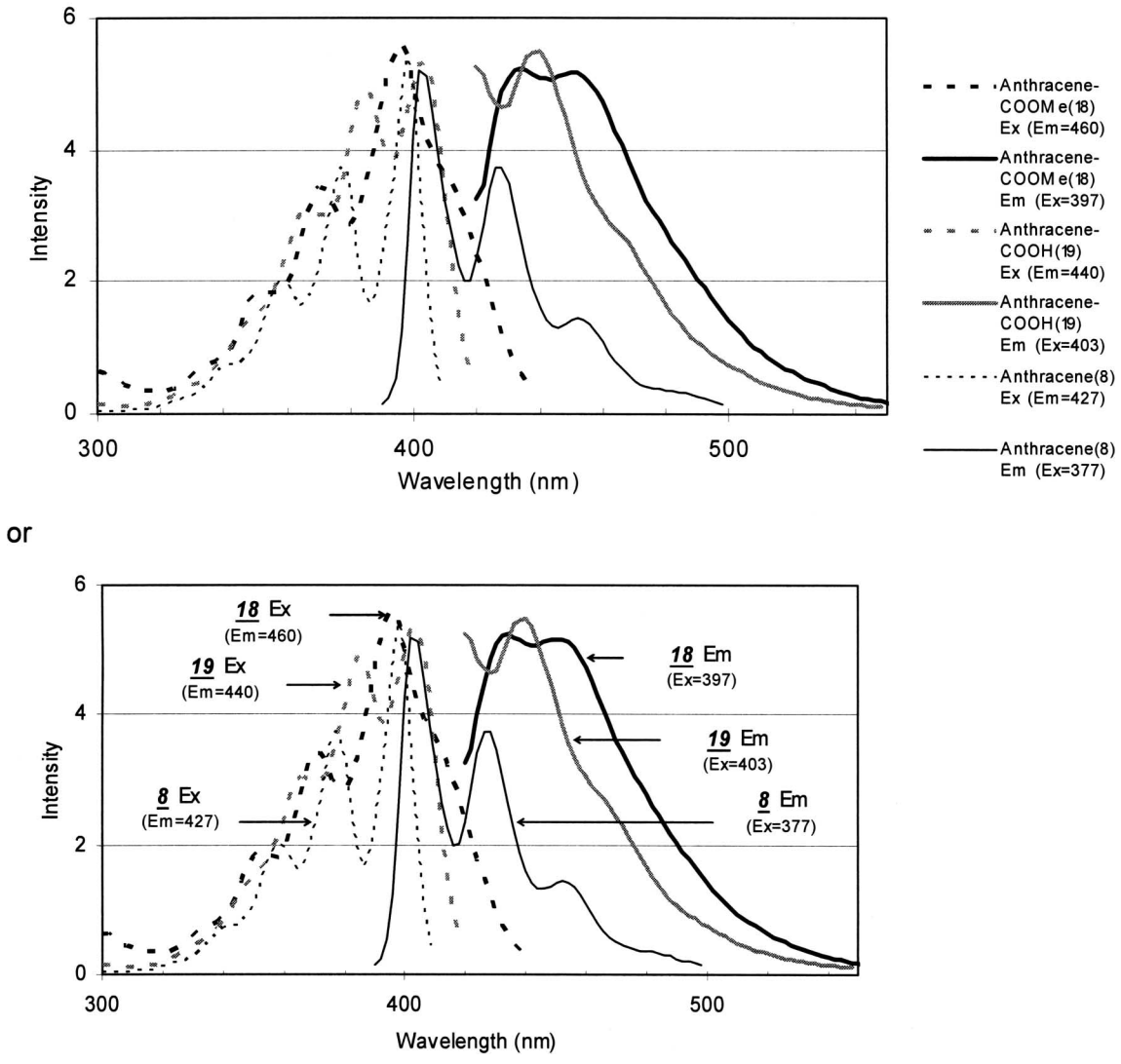


Fig. 5. Fluorescence spectra of carboxylanthracene bis-phenylboronate (**19**), carboxylanthracene bis-phenylboronate metylester (**18**), and anthracene bis-phenylboronate/double-arm (**8**) in 33% MeOH/PBS; dye concentration: 0.01 mM, instrument: SLM4800 (HV = 750, Gain = X10).

the contrary, the maximum relative fluorescence intensity changes of bis-phenylboronate type (**8**, **13**, and **16**) reached more than ten times the baseline fluorescence intensity at zero glucose concentration. Glucose response of acetyl dye (**13**) doubled compared to that of non-substituted dye (**8**). This result indicates that the introduction of the acetyl group not only improves fluorescence properties but also increases the fluorescence intensity response. The curve of the carboxylic acid dye (**19**) was similar to that of non-substituted dye.

Unfortunately, all the obtained glucose curves in solutions exhibited saturated fluorescence intensity changes by the time the glucose concentration range reached 100 mg/dL. This sensitivity is too extreme and is not suitable for measuring physiological blood glucose concentrations. However, sensitivity reductions that occur due to immobilization could result in an advantage by shifting the curve more into the physiologic range.

Glucose Sensing Performances of Sensor Membranes (Immobilized Dyes)

1st type sensor gave a poor glucose response as well as the result observed in non-immobilized study

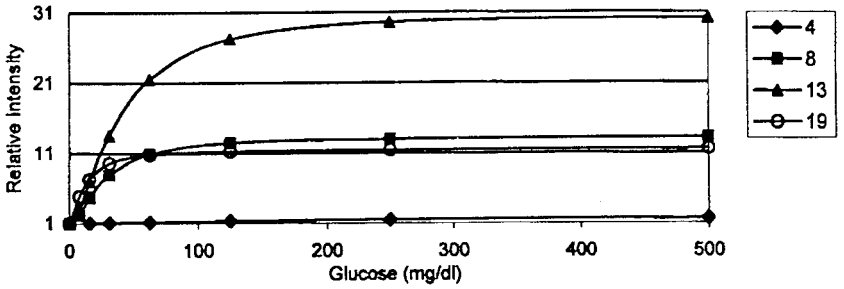


Fig. 6. Glucose response curves of anthracene mono-phenylboronate/single-arm carboxyl terminated (**4**); Ex 390 nm/Em 410 nm, anthracene bis-phenylboronate/double-arm amino terminated (**8**); Ex 377 nm/Em 427 nm, acetylanthracene bis-phenylboronate/double-arm amino terminated (**13**); Ex 400 nm/Em 480 nm, and carboxlylanthracene bis-phenylboronate (**19**); Ex 403 nm/Em 443 nm, in 33% MeOH/PBS; dye concentration: 0.01 mM, instrument: SLM4800 (HV = 750, Gain = X10).

(Fig. 6, **4**). The sensitivity of 2nd type sensor would be permissible level for commercial glucose sensor, even though drastic decline of intensity change after immobilization was observed from 30 to 1.5 in relative intensity at 500 mg/dL glucose concentration (Fig. 7). 3rd type sensor showed a quite better sensitivity compared to the previous two sensors. The sensor gave an excellent curve in the range of physiological glucose concentration in blood (62.5–500 mg/dL). Relative fluorescence intensity change reaches 2.2 at 500 mg/dL glucose concentration. The sensor performance of bis-phenylboronate/single-arm sensor (3rd type) is superior as never before (Fig. 7).

Three different coupling types were studied for immobilization of bis-phenylboronate/double-arm dye (**8**). Glucose response curves of these sensors are shown in Fig. 8. Each sensor gave different response curves. This result might be related to mobility of dye molecules, because **S-2**, which possesses the longest spacer contributing an enhancement of its mobility had the best sensitivity. In comparison with glucose curves of dyes themselves, the glucose sensitivity after immobilization fell drastically as well as for the other sensors. However, fluorescence spectra of the all dyes were not changed significantly between before and after immobilization.

It is likely that glucose sensitivity has a connection with mobility of a dye molecule on the solid phase. The double-arm immobilization includes two arms branching off from two nitrogen atoms that participate in photo-induced electron transfer. Since these arms are located close to the glucose-binding moiety, they could hinder glucose binding by reducing the mobility of a glucose-binding site. This may cause lower glucose sensitivity. On the other hand, as for the single-arm immobilization, only one arm branches from anthracene ring and is attached to the solid phase. It would not interfere with glucose detection much, because the arm is located far from the glucose-binding site. Besides that, the single arm provides free rotation for the dye, which may contribute to increased frequency of interaction with glucose molecules.

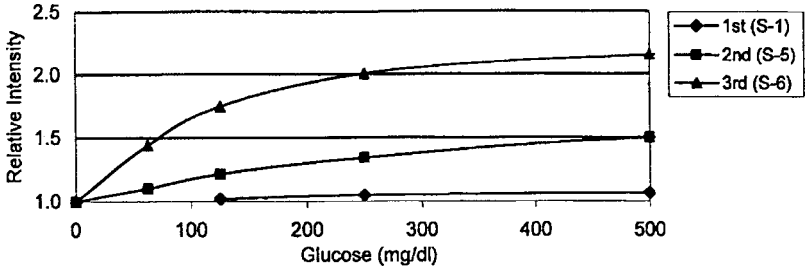


Fig. 7. Glucose curves of the sensors, which consist of 1st generation; anthracene mono-phenylboronate/single-arm (**S-1**); Ex 390 nm/Em 420 nm, 2nd generation; acetylanthracene bis-phenylboronate/double-arm (**S-5**); Ex 420 nm/Em 450 nm, and 3rd generation; anthracene bis-phenylboronate/single-arm (**S-6**); Ex 405 nm/Em 450 nm, immobilized to cellulos membrane, in PBS (pH = 7.4), instrument: SLM4800 (HV = 850, Gain = X100).

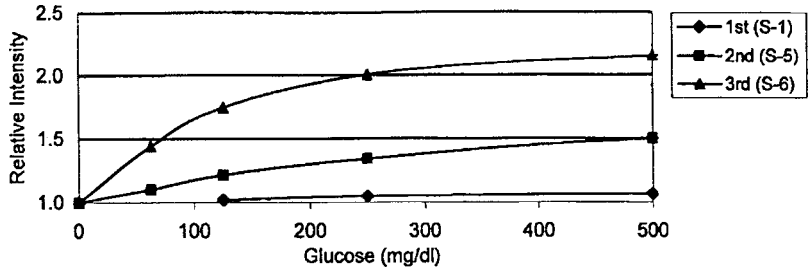


Fig. 8. Glucose curves of acetylanthracene bis-phenylboronate/double-arm (**8**) with regenerated cellulose membrane immobilized by different immobilization forms, amide coupling with long arm (**S-2**); cyanogenbromide coupling—short arm (**S-3**); medium arm (**S-4**), in PBS (pH = 7.4), Ex 377 nm/Em 425 nm, instrument: SLM4800 (HV = 850, Gain = X100).

CONCLUSION

Prior efforts to link boronate-dye glucose sensitive molecules to solid phase were done via the nitrogen atoms linking the boronate to the dye, and thus led to bilateral attachment (2nd generation). Immobilization of these boronate-fluorescent dye moieties to solid phase membranes was direct to the dye, resulting in single point attachment (3rd generation). The concept not only provided to superior glucose sensitivity, but favorably improved the fluorescence characteristics of enhanced red-shift and Stokes shift spectral properties—an obvious commercial advantage of a more simple & inexpensive optical detection system. Future sensor dye structure and membrane designs will be optimized for further improvements in sensor performance, hopefully, moving closer to the realization of a clinically usable continuous glucose monitoring system. Such an implantable continuous glucose monitor using fluorescent membranes may be less-painful yet offer high-precision blood-glucose management for diabetic patients. The technologies may also be applied to development of an artificial pancreas, e.g., a continuous sensor combined with an insulin pump.

REFERENCES

1. C. Mayerhoff, F. J. Mennel, F. Sternberg, and E. F. Pfeiffer (1996). Current status of the glucose sensor. *Endocrinologist* **6**, 51.
2. J. Pickup (1993). Developing glucose sensors for *in vivo* use. *Trends Biotechnol*. **11**, 285.
3. G. Reach and G. S. Wilson (1992). Can continuous glucose monitoring be used for the treatment of diabetes? *Anal. Chem.* **64**(6), 381A.
4. K. Tsukagoshi and S. Shinkai (1991). Specific complexation with mono- and disaccharide that can be detected by circular dichroism. *J. Org. Chem.* **56**, 4089.
5. T. D. James, K. R. A. S. Sandanayake, and S. Shinkai (1994). A glucose-selective molecular fluorescent sensor. *Angew. Chem. Int. Ed. Engl.* **33**(21), 2207.
6. T. D. James, K. R. A. S. Sandanayake, and S. Shinkai (1994). Novel photoinduced electron-transfer sensor for saccharides based on the interaction of boronic acid and amine. *J. Chem. Soc. Chem. Commun.* 477.
7. T. D. James, K. R. A. S. Sandanayake, R. Iguchi, and S. Shinkai (1995). Novel saccharide-photoinduced electron transfer sensors based on the interaction of boronic acid and amine. *J. Am. Chem. Soc.* **117**, 8982.
8. T. D. James, H. Shinmori, M. Takeuchi, and S. Shinkai (1996). A saccharide 'sponge.' Synthesis and properties of a dendritic boronic acid. *J. Am. Chem. Soc. Chem. Commun.* 705.
9. T. D. James, K. R. A. S. Sandanayake, and S. Shinkai (1995). Chiral discrimination of monosaccharides using a fluorescent molecular sensor. *Nature* **374**, 345.
10. K. R. A. S. Sandanayake, S. Imazu, T. D. James, M. Mikami, and S. Shinkai (1995). Molecular fluorescence sensor for saccharides based on amino coumarin. *Chem. Lett.* 139.
11. T. D. James, P. Linnane, and S. Shinkai (1996). Fluorescent saccharide receptors: A sweet solution to the design, assembly and evaluation of boronic acid derived PET sensors. *J. Chem. Soc. Chem. Commun.* 281.
12. M. Takeuchi and S. Shinkai (1996). Saccharide sensing using a boronic-acid probe. *Protein Nucl. Acid Enzyme* **41**(16), 2584.
13. M. Yamamoto, M. Takeuchi, and S. Shinkai (1998). Molecular design of a PET-based chemosensor for uronic acids and sialic acids utilizing a cooperative action of botonic acid and metal chelate. *Tetrahedron* **54**, 3125.
14. C. R. Cooper and T. D. James (1998). Selective fluorescenece signalling of saccharides in their furanose from. *Chem. Lett.* 883.
15. J. C. Norrild and H. Eggert (1995). Evidence for mono- and bisdentate boronate complexes of glucose in the furanose form. Application of ^{1}Jc-c coupling constants as a structural probe. *J. Am. Chem. Soc.* **117**, 1479.
16. J. C. Norrild and H. Eggert (1996). Boronic acids as fructose sensors. Structure determination of the complexes involved using 1JCC coupling constants. *J. Chem. Soc. Perkin Trans.* **2**, 2583.
17. H. Eggert, J. Frederiksen, C. Morin, and J. C. Norrild (1999). A new glucose-selective fluorescent bisboronic acid. First report of strong α-furanose complexation in aqueous solution at physiological pH. *J. Org. Chem.* **64**, 3846.
18. S. Arimori, M. L. Bell, C. S. Oh, K. A. Frimat, and T. D. James (2001). Modular fluorescence sensors for saccharides. *J. Chem. Soc. Chem. Commun.* 1836.
19. J. H. Satcher, Jr., S. M. Lane, C. B. Darrow, D. R. Cary, and J. A. Tran (2001). Glucose sensing molecules having selected fluorescent properties. WO01/20334.
20. G. T. Hermanson, A. K. Mallia, and P. K. Smith (1995). *Immobilized Affinity Ligand Techniques*, Academic Press, San Diego.
21. A. O. Patil, D. N. Schulz, and B. M. Novak (1998). *Functional Polymers*, American Chemical Society. Washington, DC.
22. K. Takemoto, R. M. Ottenbrite, and M. Kamachi (1997). *Functional Monomers and Polymers,* Marcel Dekker, New York.
23. F. Z. Dorwald (2002). *Organic Synthesis on Solid Phase*, Wiley-VCH, Verlag, GMbH, Weinheim.