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Development of tetraphenylethylene-based fluorescent oligosaccharide probes for detection of influenza virus

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ABSTRACT

Tetraphenylethylene (TPE) derivatives have strong fluorescence in aggregated state. We designed and synthesized a tetraphenylethylene derivative bearing alkyne groups which were used for combination by click chemistry. The new TPE compound bearing alkyne groups was used to synthesize fluorescence oligosaccharide probes which have lactosyl and 6′-sialyllactosyl moieties as ligands. We found that the TPE compounds bearing lactosyl and 6′-sialyllactosyl moieties were useful for detection of RCA120 and SSA lectins, respectively. Moreover, we have shown that TPE-based fluorescent oligosaccharide probe bearing 6′-sialyllactose moiety can be utilized as a "turn-on" fluorescent sensor for detection of influenza virus.

1. Introduction

A biosensor is a device for detection of an analyte using the molecular recognition mechanism of organisms. Since the first biosensor was developed by Updike and Hicks [1], a variety of biosensors have been developed [2]. Recently, Tong et al. have discovered the novel aggregation-induced emission (AIE) phenomenon [3] and applied it to biosensor technology. Among the AIE dyes, tetraphenylethylene (TPE) is synthesized by simple reactions and emits very efficiently in aggregated or crystal states. The water-soluble cationic TPE derivatives can be used as bioprobes for detection of proteins, DNA, and RNA [4]. The TPE compounds bearing adenine and thymine moieties can be used as chemosensors for Ag^{+} and Hg^{2+}, respectively [5]. Moreover, sugar-phosphole oxide conjugates were synthesized as "turn-on" fluorescent sensors for lectins [6].

Since AIE system makes it possible to detect intermolecular bindings by fluorescence signals, we envisioned that this new biosensor using AIE systems may be useful for determination of the recognition specificity of xenobiotics (bacteria, virus, and protein) for cell surface receptors. The cell surface oligosaccharides act as receptors for bacteria, virus, toxin, and other cells (a white blood corpuscle, a cancer cell). The sialyllacto/sialylneolacto-series sugar chains in glycoproteins and glycolipids are the functional receptor sugar chains for influenza A virus of humans and animals [7,8]. In addition, orthomyxovirus, polyomavirus, reovirus, coronavirus, paramyxovirus, parvovirus, adenovirus-associated virus, herpesvirus, flavivirus, and norovirus are known as viruses binding to oligosaccharides [9].

Influenza is one of the most common existing infectious diseases to both human and animal in the world. Recently, an influenza virus causes a global outbreak that becomes a menace to humanity. The receptor binding specificity of hemagglutinin, which is a viral membrane protein to attach cell surface, varies differently depending on the relation between hosts and viruses. Human influenza virus binds to the Neu5Ac2,6Gal sequence, while equine and avian viruses specifically bind to Neu5Ac2,3Gal. Porcine virus, which is called "New Influenza A (H1N1)", bind both to both Neu5Ac2,6Gal and Neu5Ac2,3Gal [10–13].

It is known that bacterial enterotoxins bind to the receptor molecules on the host cell surface. The cell surface receptors are glycoconjugates, especially glycosphingolipids. For example, chorela toxin to ganglioside GM1, *Escherchia coli* heat-labile enterotoxin to ganglioside GD1b, and Shiga toxins to Gb3Cer are known. Oligosaccharides are key molecules for development of the technology to analyze toxin–ligand interaction [14–17].

In this paper, we developed a simple method for the preparation of fluorescence oligosaccharide probes, which is a "turn-on" type sensor using tetraphenylethylene derivative, for detection of virus, toxin, and lectin. The merit of method for preparation of these probes is to be able to introduce ligands to probe compounds by "click chemistry", a Cu(I)-catalyzed azide-alkyne cycloaddition method, and can facilitate the manufacture of various biosensors. This reaction is very suitable for the preparation of various kinds

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of probes because it has high efficiency and proceeds in a variety of solvents, including aqueous alcohol or organic co-solvent and water [18]. Furthermore, using the fluorescent oligosaccharide probes bearing 6′-sialyllactose moiety, we could detect human influenza A virus.

2. Materials and methods

2.1. Reagents

Lectins were purchased from Funakoshi (Tokyo, Japan) or Seikagaku Biobusiness (Tokyo, Japan). All chemical reagents were purchased from Sigma (St. Louis, MO), Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). Nitrocellulose membrane (pore size = 0.2 μm) was purchased from Bio-Rad (Hercules, CA). Virus suspension was prepared from influenza virus-infected Madin–Darby canine kidney (MDCK) cells [19]. Briefly, MDCK cells were infected with A/WSN/33 strain at the multiplicity of infection of 0.1 in minimal essential medium containing 0.1% BSA, 1× MEM vitamin solution (Gibco), and 1 μg/ml trypsin. After 48 h post infection, the culture fluid was collected and stored at −80 °C until use.

2.2. Apparatus

^{1}H- and ^{13}C NMR spectra were measured using a 600-MHz JEOL spectrometer (JEOL, Tokyo, Japan). Electrospray ionization mass spectrometry (ESI-MS) used was a HCTultraTM (high-capacity ion trap mass spectrometer, Bruker Daltonics, Bremen, Germany). Matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF/MS) were obtained using an AutoFlex MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). The photographs under UV illumination were taken by using a FAS-III UV-image analyzer (Toyobo, Osaka, Japan). Fluorescence intensity and spectrum were measured on a Varioskan plate reader (Thermo Fisher Scientific, Waltham, MA).

2.3. Synthesis of TPE derivative compound 1

This reaction was performed following the procedures described in the literature [5]. A suspension of $TiCl_4$ (0.78 ml, 7.0 mmol) and Zn dust (0.92 g, 14.0 mmol) in 35 ml of dry THF was refluxed under N_2 atmosphere for 2 h. A solution of 4,4′-dihydroxybenzophenone (1.50 g, 7.0 mmol) in dry THF (15 ml) was added to the suspension of the titanium reagent, and the reaction was allowed to proceed at reflux for 4 h. The reaction mixture was cooled to 25 °C, poured into a 10% aqueous K_2CO_3 solution (50 ml), and after vigorous stirring for 5 min, the dispersed insoluble material was removed by vacuum filtration using Celite pad. The organic layer was separated, and the aqueous layer was extracted three times with ethyl acetate (25 ml). The combined organic fractions were washed with water and dried over $MgSO_4$.The solvents were removed in vacuo to afford the compound 1. The crude product was purified by a silica gel column using hexane-ethyl acetate (1:1, v/v) as eluent. Compound 1 was obtained in 29% yield (0.40 g). ^{1}H NMR (CD_3OD, 600 MHz): δ 6.79 (d, 8H), 6.78 (d, 8H); ^{13}C NMR (CD_3OD, 600 MHz): δ 155.3, 138.3, 136.1, 132.4, 114.0.

2.4. Synthesis of TPE derivative compound 2

The mixture of compound 1 (0.1 g, 0.25 mmol), 3-bromo-1-propyne (0.15 g, 1.25 mmol) and K_2CO_3 (0.35 g, 2.5 mmol) in anhydrous DMF (5 ml) was stirred vigorously under N_2 atmosphere at room temperature for 24 h. Then the solution was extracted with chloroform. The organic layer was washed successively with water and dried over anhydrous Na_2SO_4. After evaporation of the solvents, the crude product was purified by a silica gel column using hexane-dichloromethane (1:1, v/v) as eluent. Compound 2 was obtained in 66% yield (0.09 g). ^{1}H NMR ($CDCl_3$, 600 MHz): δ 6.93 (d, 8H), 6.70 (d, 8H), 4.62 (s, 8H), 2.51 (s, 4H); ^{13}C NMR ($CDCl_3$, 600 MHz): δ 156.1, 138.7, 137.5, 132.6, 114.1, 78.7, 75.5, 55.9; ESI-MS: *m*/*z* 548.2 ([M+H]$^+$: 549.207).

2.5. Preparation of 12-azidododecyl -lactoside and 12-azidododecyl 6′-sialyllactoside

12-Azidododecyl β-lactoside was prepared as described in literature [20]. The addition of α2,6-linked sialic acid to 12-azidododecyl β-lactoside was performed using α2,6-sialyltransferase from *Photobacterium damselae* JT160 (ST6, Japan Tobacco, Tokyo, Japan). Fifty microliters of 100 mM 12-azidododecyl β-lactoside in DMSO and 1.95 ml reaction buffer (20 mM Bis-Tris, pH 6.0, containing 0.02% Triton-X100) were mixed thoroughly. Then, 100 μl of 50 mg/ml CMP-NeuAc solution in reaction buffer and 0.1 unit ST6 was added to the solution, and the solution was incubated at 30 °C for 16 h. The crude product was purified by the SAX cartride (GL Science, Tokyo, Japan) as described previously [21]. The eluate was desalted using a C18 Sep-Pak cartridge (Waters, Milford, MA) and the solvents were removed in vacuo.

2.6. Introduction of oligosaccharides to TPE derivative

A reaction solution containing10 mM 12-azidododecyl β-lactoside (or 12-azidododecyl 6′-sialyllactoside), 2.5 mM compound 2, 10 mM $CuSO_4{\cdot}5H_2O$ and 50 mM sodium ascorbate in H_2O–DMSO(1:1, v/v) was stirred at room temperature for 24 h. The crude product was purified by silica gel column chromatography (Purif-pack SI-15, Moritex, Tokyo, Japan) using chloroform–methanol–H_2O (5:4:1, v/v/v) as eluent. As mentioned above, two kinds of fluorescence oligosaccharide probes bearing lactosyl (Lac-TPE) and 6′-sialyllactosyl (α2,6SL-TPE) moieties were manufactured.

2.7. Analysis of reactivity against lectins by dot blotting

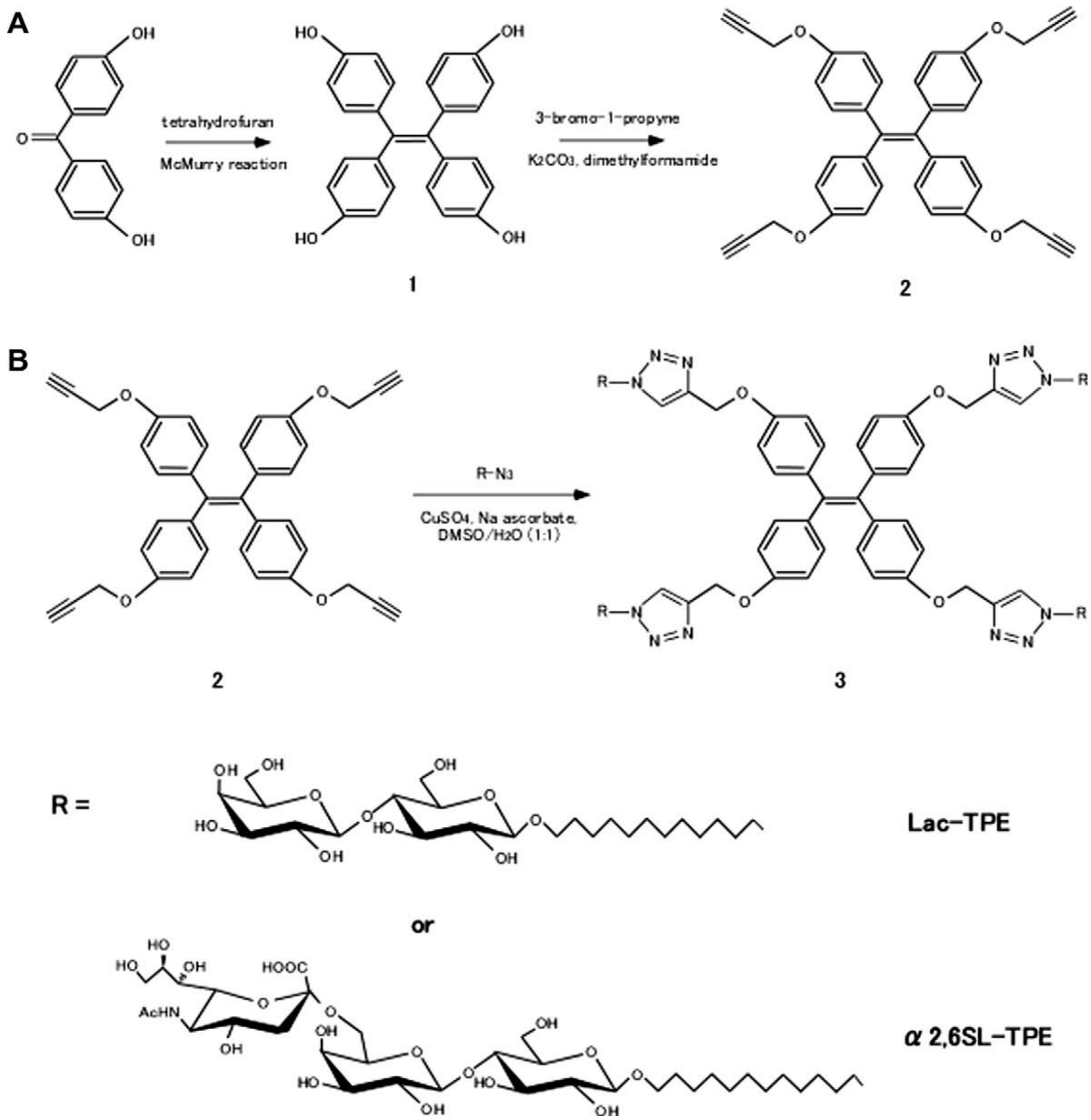
Lectins (10 μg) were spotted on nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) and dried up. Then, the membranes were soaked in 20 μM Lac-TPE/α2,6SL-TPE solution in 10 mM Tris–HCl, pH 7.6. After 10 min of exposure to the probe solutions the membranes were washed with 10 mM Tris–HCl, pH 7.6 and detected under UV illumination.

2.8. Fluorescence measurement

Fluorescence intensity was measured on a Varioskan plate reader with $\lambda_{ex}/\lambda_{em}$ set at 319/460 nm. Solutions of influenza virus and fluorescence probe were mixed in a 96-well microtiter plate. After incubation at room temperature for 10 min, fluorescence intensity and spectrum were measured.

3. Results and discussion

The TPE derivatives were prepared as shown in Scheme 1A. Compound 1 was synthesized by a McMurry coupling reaction [22] between two molecules of 4,4′-dihydroxybenzophenone. Compound 1 was converted to tetrapropargyl compound 2 by reaction with 3-bromo-1-propyne in the presence of K_2CO_3 in acceptable yield. The chemical structures and molecular masses of compounds 1 and 2 were confirmed from their NMR and mass spectra. Lactosyl and 6′-sialyllactosyl moieties were introduced into compound 2 by click chemistry between the propargyl group



Scheme 1. Synthesis of fluorescence oligosaccharide probes. (A) Synthesis of compounds 1 and 2. (B) Copper(I)-catalyzed synthesis of Lac-TPE and α2,6SL-TPE.

of TPE derivative and the azide group of aglycon of oligosaccharide compounds (12-azidododecyl β-lactoside and 12-azidododecyl 6′-sialyllactoside) using $CuSO_4$ and sodium ascorbate as shown in Scheme 1B. 12-Azidododecyl β-6′-sialyllactoside was obtained by enzymatic reaction from 12-azidododecyl β-lactoside using ST6 in 70% yield. Because the solubility of TPE derivative and12-azidododecyl β-lactoside to water was low, they were dissolved in DMSO and added to the reaction solution. TLC analysis (chloroform–methanol–H_2O, 5:4:1) of these mixtures showed no sign of the starting compound 2. Analysis of the MALDI-TOF mass spectrum of Lac-TPE and α2,6SL-TPE revealed a peak at *m*/*z* 2777.6 ($[M+Na]^+$) and 4030.9 ($[M-4H+5Na]^+$), respectively.

By dot blot method using lectin we confirmed the binding specificity of two kinds of fluorescent oligosaccharide probes (Lac-TPE and α2,6SL-TPE). Lectins RCA120 and SSA are known to bind specifically to lactose and sialyl(α2,6)Gal/sialyl(α2,6)GalNAc sequence, respectively. Lac-TPE and α2,6SL-TPE bound to RCA120 and SSA, respectively (Fig. S1). These fluorescent oligosaccharide probes were confirmed to be useful tools for detection by dot blot method. It is usually necessary for the analytes to have plural bind-

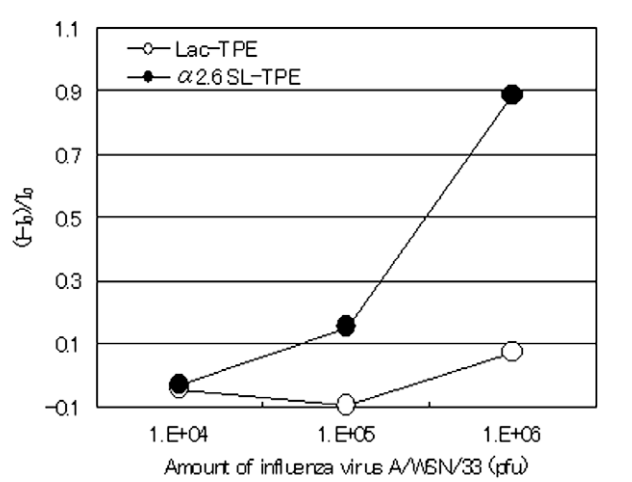


Fig. 1. Fluorescence intensity at 460 nm of Lac-TPE and α2,6SL-TPE (2.5 μM) in the presence of different concentrations of influenza virus A/WSN/33 in 10 mM Tris–HCl, pH 7.6.

ing sites to form aggregate states in the solution. However, the analytes immobilized on the membranes were detectable even if they have only one binding site.

Human influenza viruses bind preferentially to sialic acids linked to galactose by an α2,6 linkage. We examined whether influenza virus could be specifically detected by the fluorescence oligosaccharide probe, because the influenza virus possesses HA molecules at a high density (about 1000 molecules/virus) on its surface [23]. Fig. 1 shows the fluorescence spectrum of Lac-TPE and α2,6SL-TPE in the presence of different concentrations of influenza virus A/WSN/33 in 10 mM Tris–HCl, pH 7.6. Fluorescence enhancement of α2,6SL-TPE was observed in the presence of influenza virus. On the other hand, fluorescence of Lac-TPE was not enhanced in the presence of influenza virus. These results suggest that AIE may have been caused by binding of influenza virus to 6'-sialyllactose moiety ligated to α2,6SL-TPE. It is interesting that whether the TPE derivatives bind to one virus particle or viral aggregates to induce such fluorescence enhancement by AIE effect. This AIE effect is mainly caused by the restriction of intramolecular rotation of C–C single bond [24]. The α2,6SL-TPE may bind to the HA molecules on the surface of an influenza virus and freeze its intramolecular rotation.

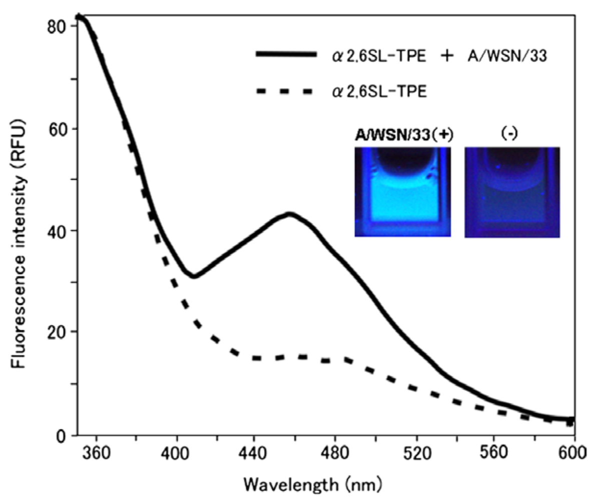


Fig. 2. Fluorescence spectrum and photographs of α2,6SL-TPE (5 μM) in the presence (10^6 pfu) or absence of influenza virus A/WSN/33 in 10 mM Tris–HCl, pH 7.6.

Fig. 2 shows the fluorescence spectrum of α2,6SL-TPE in the absence and presence of influenza virus A/WSN/33 in 10 mM Tris–HCl, pH 7.6. α2,6SL-TPE showed very feeble emission at the concentration of 5 μM. The emission of less than 400 nm light depends on the self-luminosity of the 96-well microtiter plate. However, after addition of influenza virus A/WSN/33, the emission band at 460 nm increased. Actually, the difference of fluorescence between the absence and presence of influenza virus can be distinguished by the naked eye under UV illumination as shown in Fig. 2. Moreover, we examined detectable amounts of influenza virus. The fluorescence intensity of α2,6SL-TPE at 460 nm increased significantly as a function of increasing amounts of influenza virus as shown in Fig. 3. The influenza virus with a concentration higher than 10^5 pfu/100 μl was significantly detected.

It is well-known that influenza viruses bind to sialyl sugar chain receptors on host cell membranes. Trimeric hemagglutinin (HA) molecules on the surface of the envelope membrane of the virus plays an important role in influenza virus infection to human cells. The binding specificity of HA changes easily by a mutation of the *HA* gene (only two sets of amino acid exchange) [13], so that the detection method of influenza virus using oligosaccharide probes is quite useful for diagnosis of the influenza virus.

In conclusion, we developed a simple method for the preparation of fluorescence oligosaccharide probes. TPE derivatives with propargyl residues could be used for ligation to oligosaccharide compounds with azide residues by click chemistry. Because the ligation reaction proceeded with $CuSO_4$/ascorbate in water/DMSO mixtures at room temperature, water-soluble compounds such as oligosaccharide could be easily ligated to TPE derivatives. By this technique, we prepared fluorescence oligosaccharide probes and demonstrated that α2,6SL-TPE can be used as fluorescent sensor for influenza virus.

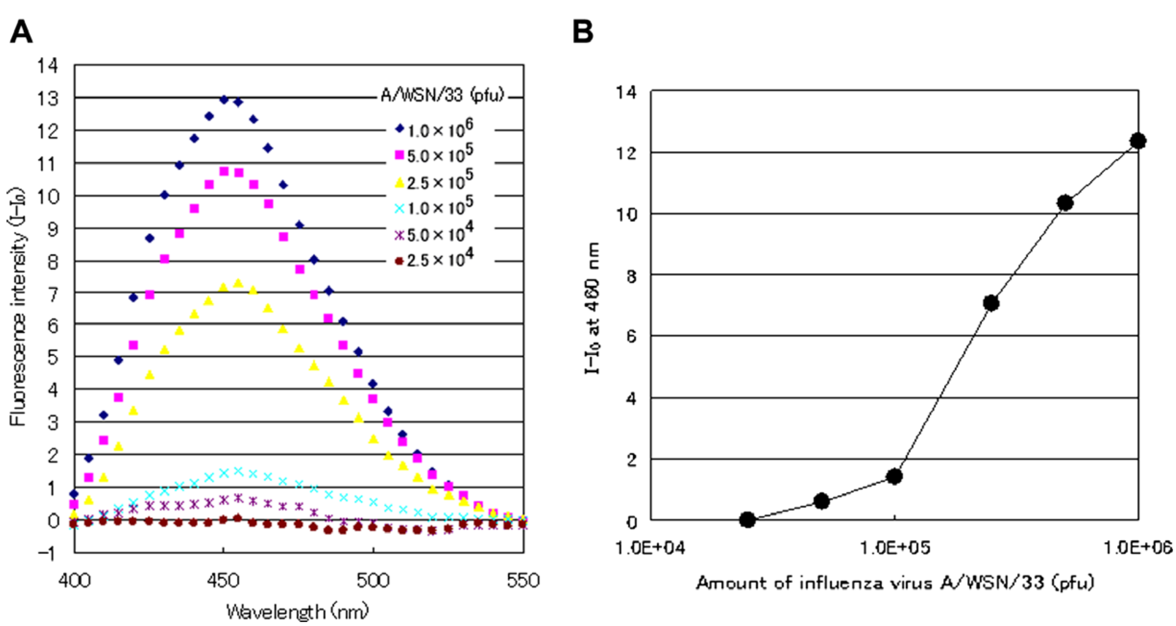


Fig. 3. Change in (A) fluorescence spectrum and (B) intensity of α2,6SL-TPE (5 μM) after addition of influenza virus A/WSN/33 in10 mM Tris–HCl (pH 7.6). I_0 is the value in its absence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.155.

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