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A highly sensitive probe detecting low pH area of HeLa cells based on rhodamine B modified β-cyclodextrins

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ABSTRACT

Two kinds of rhodamine modified β-cyclodextrins (R-1 and R-2), which are coupled up ethylene diamine (EDA) and tetraethylene pentamine (TEPA) between Rh B and β-cyclodextrin, respectively, have been synthesized. R-1 and 2 work as a new fluorogenic probe for monitoring pH of Hela cells, and MTT of assay R-1, R-2, and rhodamine B indicate that less a cytotoxicity of those R-1 and R-2 than that of rhodamine B, where R-1 has much less one than that of R-2. The fluorogenetic probe capability of R-2 was recognized in an area of acidic area in living cell, which is lysosome.

1. Introduction

Cyclodextrins (CDs) are tours shaped cyclic oligosaccharides composed of six, seven and eight D-glucopyranose units (α, β, γ-CDs, respectively). A variety of organic compounds can be included in their central cavities in aqueous solution.[1] Because of nontoxic property of CDs based on non-permeability of biological membrane, CDs are utilized in so many fields such as inclusion of molecules, DDS of anticancer and host as spectroscopy study in addition of chemical sensor detecting for organic molecules.[2–6] The pharmaceutical approach of CD is based on inclusion functionality and extended-release capability. On the other hand, modified CD such as fluorescent modified CD (Fl-CD) has been used as molecular sensing probe for organic molecules,[7,8] because fluorescent moiety works as probe as fluorescence emission analysis and CD works as a cavity for gust molecules. Unfortunately, Fl-CD has not been used as pharmaceutical application such as DDS carrier. It is expected that amphiphile of Fl-CD seems to have affinity for amphilic biological membrane, where amphilic CD will be utilized as transportation medium for DDS. For this purpose, we tried to synthesize cell membrane traffic Fl-CD. In endocytotic system of a cell, there are some organelles showing acidic property. Those things are called as endosome and lysosome, where pH are 6.0 and 5.0–4.5, respectively.[9–11] Derivatives of xanthene such as fluorescein and rhodamine (Rh) dyes are widely employed as molecular probes in chemical biology, especially Rh dyes are used extensively in biotechnological applications such as fluorescence microscopy, flow cytometry, fluorescence correlation spectroscopy and ELISA, because Rh dyes are highly fluorescent and resistant to photo-bleaching.[10–14] It is well known that derivatives of Rh molecule such as rhodamine B (Rh B) can be taken in cellular without endocytosis because those dyes have positive charge, which show affinity for cell membrane of negative charge and endoplasmic reticular. On the basis of versatile functionality of Rh dyes, we study a synthesis of Rh B modified β-CDs, which has cell membrane permeability.

2. Results and discussion

2.1. Synthesis of rhodamine B modified β-CDs (R-1 and R-2)

The synthesis of Rh B modified CDs (R-1, R-2) is shown in Scheme 1. R-1 has been synthesized as same way in only difference of using of ethylene diamine (EDA) instead of tetraethylene pentamine (TEPA). The yield of R-2 is very low in comparison with that of R-1. Because linker chain of R-2 is much longer than that of R-1, it might be happened that the long chain will be clumpy.

2.2. UV–vis spectra and fluorescence spectra of rhodamine modified β-CDs

UV–vis spectra of R-1 in different pH region were shown in Figure 1a, where a new peak at 570 nm appeared with small

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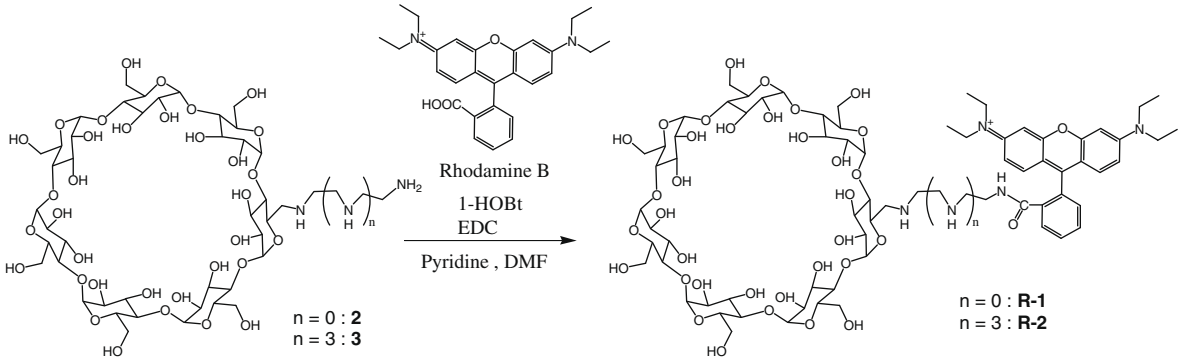

Scheme 1. Preparation of R-1 and R-2.

magnitude when R-1 was kept in pH 3.0 aqueous solution. On the other hand, the peak of R-2 at 560 nm was increased with high absorbance as shown in Figure 1b. The new peak was derived from amide form of R-2. The structural conversion of R-2 might be proceeded as shown in Figure 2, because Walter et al.[11] reported structures of the amide and lactam forms of the model compound such as rhodamine 101 conjugates, which exist in pH-depended equilibrium. Figure 3 shows time-dependent of absorbance alternation at 240 nm and 560 nm of R-2 at pH 3.0, which indicates that keeping of R-2 in pH 3.0 for 15 min alternates from lactam form to amide one completely. It means complete alternation of structure of R-2 takes 15 min. The amide form of R-2 was produced in this reaction condition. It seemed that the reaction of R-2 was carried out in basic condition because of using pyridine. Figure 4 shows the fluorescent intensity of R-2 at 560 nm in different pH region. As shown in Figure 4, high fluorescent intensity was recognized in only at pH 3.0 region, which indicates amide form of R-2 existed in under at pH 3.0 region. These results suggested that R-2 shows fluorescent switching property in different pH region. These phenomena were utilized in the biological application.

2.3. Cytotoxicity evaluation

Cytotoxicity of β-CD derivatives including native β-CD (**1**), ethylene diamine-modified β-CD (**2**), and tetraethylene pentamine-modifed β-CD (**3**) in human Jurkat cells and HeLa cells was evaluated by MTT assay[15] based on mitochondrial reduction of the yellow product. This assay usually shows high correlation with number of living cells, cell proliferation and release of mitochondrial matrix enzymes. Those results on Jurkat and HeLa cells were shown in Figure 5a and b, respectively. As shown in Figure 6a and b, cytotoxicity of Rh B, R-1 and R-2 were also investigated. It is shown that **3** shows cytotoxicity for Jurkat cells in high concentration of 1000 μM as shown in Figure 5a. For HeLa cells, **1**, **2** and **3** do not show cytotoxicity even in high concentration. The sequence of cytotoxicity of those compounds are **1**<**3**<R-1<R-2<<Rh B. MTT assay shows modification of Rh B by CD reduces its cytotoxicity, where R-1 shows much better than that of R-2 at the 100 μM. It seems that the cytotoxicity of those compounds for HeLa cells is less than that of Jurkat cells. It might be caused that a difference of surface area of those cell membrane derived from floating cell of Jurkat cells and adhesive cell of HeLa cells.

2.4. Fluorescence image of HeLa cells

A staining of living HeLa cells by R-2 was investigated. The pictures were taken by fluorescence microscope. Figure 7 shows a staining capability of Rh B for HeLa cells, where selective stainability was not recognized. On the other hand, outstanding selectivity of staining by R-2 for HeLa cells was recognized. Figure 8 shows the staining result of R-2 for HeLa cells, where granulated fluorescence substance was recognized on the fringe of the nucleus and the light spot was partially recognized around neighborhood of the membrane. It means that endoplasmic reticula area is existing in acidic condition. To confirm the selectivity of R-2, Lyso Tracker blue, which stains lysosome with high selectivity, was used, where the light spot was partially recognized around neighborhood of the membrane .as same using R-2. These results suggested that R-2 was incorporated into intracellular area by endocytosis action.

3. Conclusion

In conclusion, we declared that the fluorescent intensities of the titled compounds were affected by pH, where the lactam formation

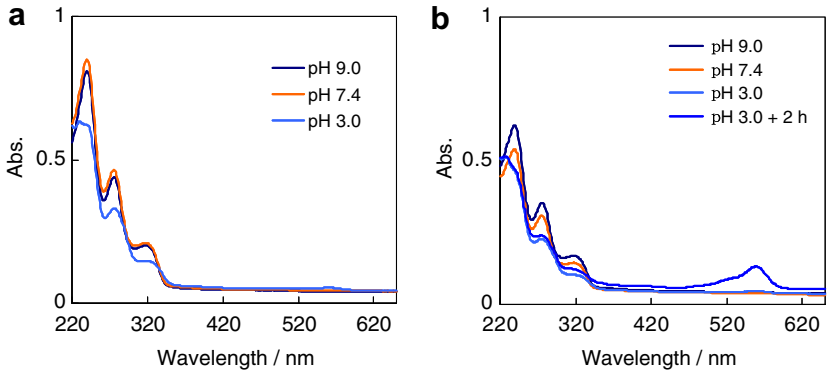

Figure 1. (a) UV spectra of R-1 at the pH 9.0 (—), 7.4 (—) and 3.0 (—). (b) UV spectra of R-2 at the pH 9.0 (—), 7.4 (—), 3.0 (—) and 3.0 + 2 h (—).

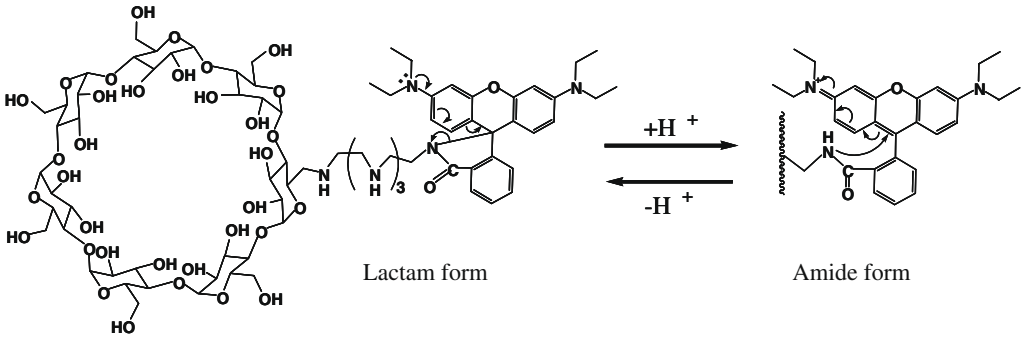

Figure 2. The structural conversion of amide and lactam form of R-2 at pH condition.

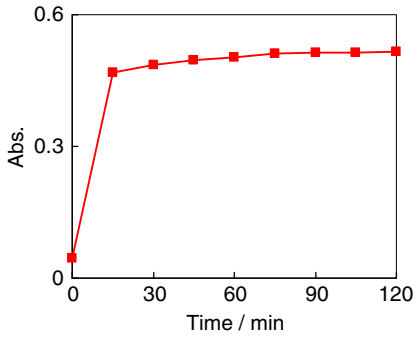

Figure 3. The time variation of absorbance intensity at the 560 nm (—) of R-2 at pH 3.0.

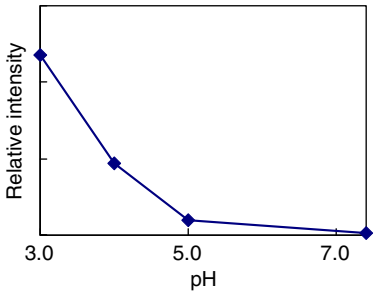

Figure 4. The pH variation of fluorescent intensity of R-2 at 560 nm. The concentration of R-2 was 30 μM, emission and excitation was 590 nm and 630 nm, slit width was 5 nm.

equilibrium would shift to charged amide form at low pH. It was shown that the intensity of R-2 was higher than that of R-1. It might be attributed to the longer length of linker because of the less affection by CD. Rh B is accumulated and kept in mitochondria of living cells, although R-1 and R-2 show different staining ability where coloring was observed in the area of outside of nucleus. That result looks like behavior of Lysotracker blue. It is known that Lysosome is low pH organelle at a region from 4.5 to 5. We assumed that the accumulation of R-1 and R-2 in lysosome was action of endocytosis. The reducing of cytotoxity of R-1 and R-2 by comparison Rh B might be attributed to not passing through cell membrane. We concluded that R-2 could be used as sensor to identify acidic area in living cell with fluorescent image.

4. Materials and methods

4.1. Materials

CDs were a gift from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). rhodamine B and 1-hydroxybenzotriazole (1-HOBt) and ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were commercially available (Tokyo Chemical Industry Co., Ltd.). Mono-6-(2-aminoethyl)-amino-6-deoxy-β-cyclodextrin and mono-[6-(11-amino-3,6,9-ethylene-amino)-6-deoxy]-β-cyclodextrin was synthesized according to the procedure reported previously.[16]

4.2. Preparation of R-1

Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 153 mg, 0.80 mmol) was added to a cooled solution

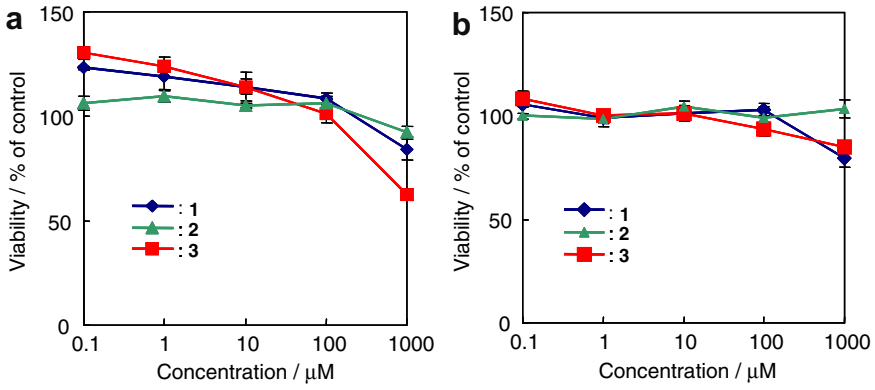

Figure 5. (a) The MTT assay of **1** (—◆—), **2** (—▲—) and **3** (—■—) with Jurkat cells. (b) The MTT assay of **1** (—◆—), **2** (—▲—) and **3** (—■—) with HeLa cells.

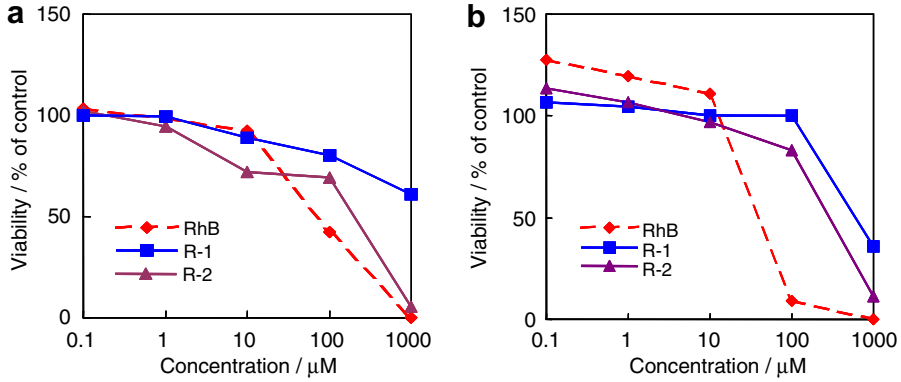

Figure 6. (a) The MTT assay of Rh B (-◆-), R-1 (—■—) and R-2 (—▲—)with Jurkat cells. (b) The MTT assay of Rh B (-◆-), R-1 (—■—) and R-2 (—▲—) with HeLa cells.

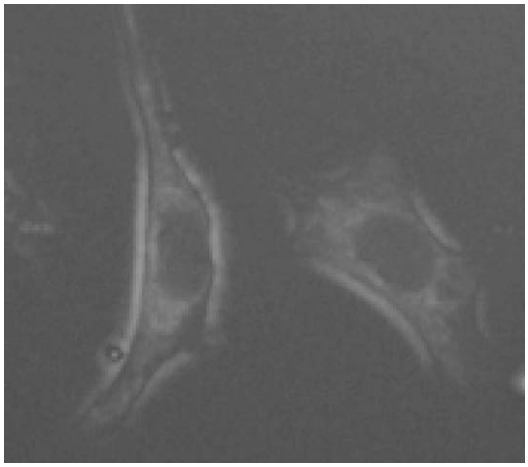

Figure 7. The picture of living HeLa cells staining of Rh B.

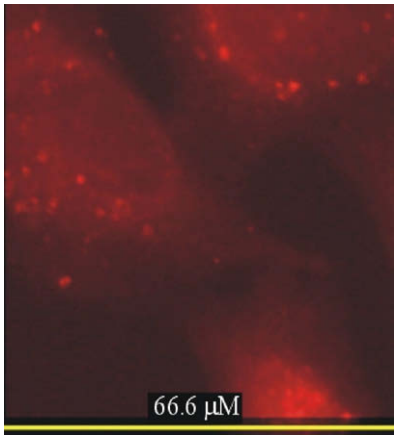

Figure 8. The picture of living HeLa cells staining of R-2.

(−20 °C) of rhodamine B (373 mg, 0.78 mmol) and 1-hydroxybenzotriazole (105 mg, 0.78 mmol) in 15 mL DMF and 10 mL pyridine. The reaction mixture was stirred at −20 °C for 2 h. To a stirred solution, mono-6-(2-aminoethyl)-amino-6-deoxy-β-cyclodextrin (500 mg, 0.39 mmol) was added portion wise. The solution was stirred for 30 min at −20 °C and then the reaction mixture was stirred at room temperature for 24 h. The solution was evaporated in vacuo to small amount and then the residue was washed with acetone (300 mL). The resulting precipitates were collected and dried under vacuum overnight, to give 590 mg crude product. The crude product was dissolved in small amount of water and loaded on column chromatography on CM Sephadex C_{50}. After removal of impurities by elution with water, the fractions containing desired product were eluted of 2.5% ammonia solution then it product were concentrated on a evaporation. Desired product was poured into acetone (300 mL), and resulting precipitates were collected and dried under vacuum overnight, to give a pink powder (770 mg, yield 20.1%). ^{1}H NMR (D_2O): δ = 7.83, 7.80 (d, d, 1H; rhodamine aromatic), 7.48 (s, br s, 2H; rhodamine aromatic), 6.79 (br s, 1H; rhodamine aromatic), 6.32–5.92 (m, 8H; rhodamine xthantan), 4.91 (s, 7H; C^1H of CD), 4.05–3.89 (m, amine CH_2), 3.93–3.09 (br s, 48H; CD C^2–C^6H, rhodamine CH_2), 2.03 (t, 4H; amine CH_2) 1.83 (t, 2H; NH), 1.21–0.76 (m, br s 12H; rhodamine CH_3) FAB MS: *m/z*: 1623.79 [$M-H^+$+Na^+] ((Calcd for $C_{72}H_{104}N_4O_{36}$: 1601.61) UV λ_{max} (H_2O) nm (log ε) 240 (5.157).

4.3. Preparation of R-2

Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 0.170 g, 0.887 mmol) was added to a cooled solution (−20 °C) of rhodamine B (373 mg, 0.78 mmol) and 1-hydroxybenzotriazole (108 mg, 0.77 mmol) in 15 mL DMF and 10 mL pyridine. The reaction mixture was stirred at −20 °C for 2 h. To a stirred solution was added portion wise, mono-[6-(11-amino-3,6,9-ethylene-amino)-6-deoxy]-β-cyclodextrin (500 mg, 0.38 mmol) in small amount of pyridine was added. The solution was stirred for 30 min at −20 °C and then the reaction mixture was stirred at room temperature for 2 days. The solution was decreased to solvent by evaporator then poured into acetone (300 mL). The precipitate was collected and dried under vacuum overnight, to give 680 mg crude product. The crude product was dissolved in water and purified by column chromatography on CM Sephadex C_{50}. After removal of impurities by elution with water, the fractions containing desired product were eluted of 2.5% ammonia solution then it product were concentrated on a evaporation. Desired product poured into acetone (300 mL). The product was collected and dried under vacuum overnight, to give a pink powder (77 mg, yield 1.16%). ^{1}H NMR (D_2O): δ = 7.82 (br s, 1H; rhodamine aromatic), 7.74 (br s, 2H; rhodamine aromatic), 6.89 (br s, 1H; rhodamine aromatic), 6.48–5.84 (m, 8H; rhodamine xthantan), 4.92 (s, 7H; C^1H CD), 4.07–2.90 (br s, 48H; CD C^2–C^6H, rhodamine CH_2), 2.57–2.25 (br s, 20H;CH_2 amine), 2.10 (t, 6H; NH_2), 1.13–0.67 (br s, 12H; rhodamine CH_3) FAB MS: *m/z*: 1752.96 [$M-H^+$+Na^+] Calcd for $C_{78}H_{119}N_7$O36: 1730.808 λ_{max} (H_2O) nm (log ε) 240 (5.040).

4.4. Methods

Measurement: Thin-layer chromatography (TLC, *n*-butanol:ethanol:water, 5:4:3, 1-propanol:30% NH_3 aq:ethyl acetate:water, 1:1:1:1) was carried out with silica gel F_{254} (Merck Co.). Fluorescent spectra were measured on a Perkin Elmer LS 50 B. Absorption of MTT assay measured by micro plate reader using Bio Rad model 550.

4.5. Cell culture

HeLa cells and Jurkat cells were cultured in Eagle's MEM and RPMI-1640, respectively, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. Exponentially growing cells cultured in a humidified chamber at 37 °C containing 5% CO_2 were used for various experiments.

4.6. Cell proliferation assay

Cell proliferation was evaluated using the colorimetric 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. HeLa cells (1×10^4 cells) were seeded in 96 well plates 24 h prior to adding 0.1% DMSO containing drugs, then were incubated an additional 48 h. After treatment, the cells received 10 μl of 5.5 mg/ml MTT and were incubated at 37 °C for 4 h. A 90-μl aliquot of extraction solution (40% *N*,*N*-dimethylformamide, 2% CH_3COOH, 20% SDS and 0.03 N HCl) was added to each well and mixed thoroughly by overnight agitation at room temperature. Cell proliferation was determined by measuring optical density at 570 nm using a Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

4.7. Fluorescence imaging

HeLa cells were cultured on 8-chamber slides for 20 h, and then incubated for 5 h in the presence or absence of the drugs. After removal of the medium, cells were fixed in 3.7% paraformaldehyde for 30 min. After washing, cells were mounted with aqueous mounting medium. Fluorescence images were observed using a fluorescent microscope IX70 (Olympus, Tokyo, Japan).

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