Binding Affinities of Carbohydrate-Conjugated Chlorins for Galectin-3

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Received October 18, 2007

Carbohydrate-conjugated chlorins were synthesized for use as biosensors for the detection of the galectin-3 cancer marker. We used ELISA, SDS-gel electrophoresis, and Bradford assays to examine the binding of galectins to D-(+)-galactose- and β-lactose-conjugated chlorins. The binding affinities of these conjugated chlorins for galectin-3 were quantified using fluorescence spectroscopy. The fluorescence emission of the carbohydrate-conjugated chlorins decreased as the amount of galectin-3 in the binding reaction increased over a limited concentration range, indicating that carbohydrate-conjugated chlorins are potentially useful fluorescence biosensors for the galectin-3 cancer marker.

Key Words : Biosensor, Cancer marker, Carbohydrate, Chlorin, Galectins

Introduction

Galectins are found in numerous types of cell and tissue. Their various functions have made them the focus of emerging research areas, not only in glycobiology, but also in medicine.[1] Galectins play particularly intriguing roles in cancer.[1,2] Both intra- and extracellular galectins act in processes as diverse as apoptosis,[3] metastasis,[4] and angiogenesis.[5] In addition to playing roles in cancer, their effect on the immune system is a major source of inspiration for scientists.[6] Combined efforts have mobilized chemists to develop new galectin-based compounds with therapeutic potential and with possible application in further deciphering the biological roles of galectins. Simple, cheap, and accurate methods to detect and quantify the amounts of the various galectins within tissues are also needed.[7]

Galectins bind to sugar molecules on the surface of cells. All galectins bind lactose and other β-galactosides, but differ in their affinities for more complex saccharides.[8] Thus far, 14 mammalian galectins have been identified, all of which harbor a conserved carbohydrate recognition binding domain (CRD) of approximately 130 amino acids. Of these galectins, galectin-3, which is a ubiquitous protein with a variety of biological roles, is the only one of the vertebrate chimera types; it contains one CRD connected to an unusually long N-terminal proline- and glycine-rich domain.[9]

Galectin-3 is the most studied member of the galectin family. Its biological functions were initially attributed to its carbohydrate-binding activity, but over the past decade, a whole new spectrum of non-lectin-related galectin-3 functions has been revealed.[1] High levels of circulating galectin-3 are correlated with increased potential for malignancy in several types of cancer, and galectin-3 plays a role in tumor growth, metastasis, and cell-to-cell adhesion.[10] The amino acid sequences of human and mouse galectin-3 are approximately 80% homologous.[11]

Galectin-8, another potential human tumor antigen.[12] is a tandem repeat-type galectin comprising a single polypeptide chain with two distinct but homologous CRDs separated by a non-conserved linker sequence of up to 70 amino acids.[7] High levels of circulating galectin-8 are correlated with the presence of lung carcinomas, certain forms of prostate carcinoma, and other tumor cells.[12] It acts as a physiological modulator of cellular adhesion and growth and may be involved in neoplastic transformation.[13-15] Like galectin-3, galectin-8 is approximately 80% homologous in amino acid sequence between human and mouse.[11]

The expression of galectin-3 has recently emerged as a potential diagnostic marker of some cancers.[16] Particularly encouraging results were obtained for thyroid cancers, suggesting that the measurement of galectin-3 expression might be useful in the diagnosis of this malignancy.[17] Currently, no convenient and economical methods are available for the detection of galectins in tissue samples, although antibody-based methods such as ELISA and Western blotting are in use. A chemical probe-based method shows potential as an alternative method.[7]

In recent years, a number of carbohydrate derivatives of various photosensitizers have been synthesized for use in photodynamic therapy.[18] Because the highly conserved 15-kDa CRD of galectins has a high affinity for β-galactosides, galectins have been among the specific proteins investigated as potential markers for malignant tumors.[18] Because chlorin-based compounds exhibit both highly selective localization for cancer cells and remarkable fluorescence emission properties, they may have broad application in the diagnosis and treatment of various cancers.

We have been developing galectin-3-binding biosensors using carbohydrate-conjugated chlorin compounds. This report details various aspects of this research, including the synthesis of carbohydrate-conjugated chlorins, a solid-phase binding assay, and galectin analysis using SDS-polyacrylamide gel electrophoresis and Bradford assay. Fluorescence emission was used to quantify the binding affinities of the carbohydrate-conjugated chlorins for galectin-3 and galectin-8. Our aim was to gather comprehensive information

pertinent to the possible use of carbohydrate-conjugated chlorins as efficient nanobiosensors for the galectin-3 cancer marker.

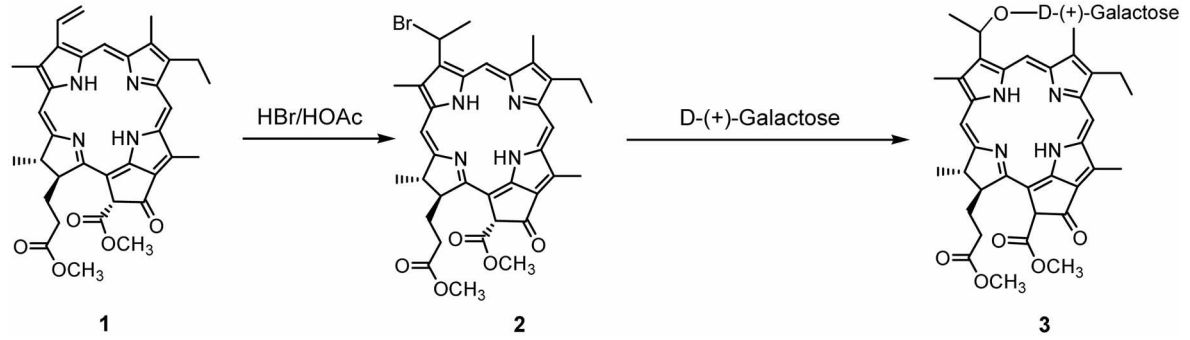
Experimental Section

Synthesis of β-D-(+)-galactose-conjugated chlorin. All chemicals were purchased from Sigma-Aldrich Corporation (Saint Louis, MO, USA) unless otherwise noted. The solvents were purified according to standard procedures. UV-visible absorption spectra were obtained using a Scinco S-3110 UV-Visible Spectrophotometer (Scinco Co., Ltd. Seoul, Korea). NMR analysis was conducted by the Research Support Team of the Korea Advanced Institute of Science and Technology (Daejeon, Korea) using a Bruker FT500 NMR (Bruker Optics Inc., Billerica, MA, USA). The synthesis of 3-[1-(D-(+)-galactopyranosyloxy)ethyl]-3-devinylpheophorbide methyl ester, also known as β-D-(+)-galactose-conjugated chlorin is illustrated in Scheme 1.

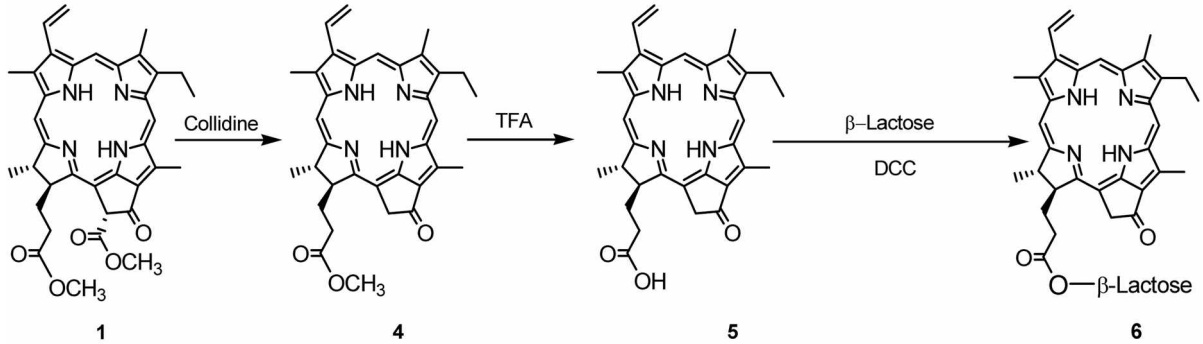
3-[1-(D-(+)-galactopyranosyloxy)ethyl]-3-devinylpheophorbide methyl ester 3. A 30% solution of HBr in acetic acid (6 mL) was added to methyl pheophorbide-a **1** (100 mg, 0.165 mmol) at 0 °C.[19] The mixture was kept in the dark at room temperature for 2 h and then evaporated *in vacuo* to yield 3-(1-bromo)-3-devinylpheophorbide-a methyl ester **2**.[20] After the resulting compound **2** (100 mg, 0.15 mmol) was dissolved in dimethyl formamide (50 mL), anhydrous K_2CO_3 (500 mg, 3.62 mmol) and D-(+)-galactose (800 mg, 4.44 mmol) were added. The reaction mixture was stirred at 80 °C for 8 h, and the solvent was removed. The residue was purified by chromatography on a silica-gel column to yield the title compound **3** (42 mg, 0.053 mmol). Yield: 36%; Mp: > 300 °C (decomp); UV-vis (CH_2Cl_2): λ_{max} (rel. int. = 1): 664.6 nm (0.51), 607.9 (0.11), 537.4 (0.12), 505.2 (0.15), 407.6 (1.51). ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 9.38, 9.19, 8.56 (each s, 1H, meso-H), 8.01 (1H, m, 3a-H), 6.23, 6.17 (each 1H, d, 3b-H), 6.30 (s, 1H, 13^2-H), 4.40 (1H, q, 18-H), 4.29 (1H, d, 17-H), 4.89-3.85 (m, Sugar-H), 3.81 (s, 3H, 15b-H), 3.67 (s, 3H, 12-Me), 3.66 (q, 8a-Me), 3.40 (s, 3H, 17-OMe), 3.24 (s, 3H, 2-Me), 2.96 (s, 3H, 7-Me), 2.56, 2.78 (m, 4H, 17a+17b-H), 1.79 (d, 3H, 18-CH_3), 1.69 (s, 3H, 8b-Me), 0.08 (each br s, NH).

Synthesis of β-lactose-conjugated chlorin. All chemicals and the instruments used for the synthesis of β-lactose-conjugated chlorin were the same as those used in the process shown in Scheme 1. The synthesis of pyropheophorbide-a lactose ester, also known as the β-lactose-conjugated chlorin is illustrated in Scheme 2.

Pyropheophorbide-a β-lactose ester 6. Methyl pyropheophorbide-a **4** (200 mg, 0.365 mmol) was dissolved in 80% aqueous trifluoroacetic acid (30 mL), and the reaction mixture was stirred at room temperature for 4 h under nitrogen.[19] The solvent was poured into cold water (200 mL) and extracted with dichloromethane (3 × 100 mL), washed with water (2 × 100 mL), and dried over anhydrous sodium sulfate. Evaporation of the solvent gave pyropheophorbide-a **5** (136.4 mg, 0.255 mmol).[21] The resulting pyropheophorbide-a **5** (50.0 mg, 0.093 mmol) was dissolved in dry dimethyl formamide (20 mL); β-lactose (30.0 mg, 0.088 mmol) and dicyclohexylcarbodiimide (40 mg, 0.19 mmol)



Scheme 1. Synthesis of 3-[1-(D-(+)-galactopyranosyloxy)ethyl]-3-devinylpheophorbide methyl ester.



Scheme 2. Synthesis of pyropheophorbide-a β-lactose ester.

were then added to the solution. The reaction mixture was stirred at room temperature for 12 h under nitrogen, and the solvent was removed *in vacuo*. The residue was purified on a silica gel column to yield the title compound **6** (17.6 mg, 0.021 mmol). Yield: 22%; Mp: > 300 °C (decomp); UV-vis (MeOH): λ_{max} (rel. int. = 1): 665.7 nm (0.09), 610.4 (0.02), 540 (0.01), 509.1 (0.01), 409.6 (0.19); ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 9.82, 9.53, 8.95 (each s, 1H, meso-H), 8.30 (1H, m, 3a-H), 6.46, 6.38 (each 1H, d, 3b-H), 5.79 (s, 1H, 13^2-H), 5.79 (1H, d, 17-H), 4.63 (1H, q, 18-H), 4.17-3.77 (m, Sugar-H), 3.77 (q, 8a-Me), 3.67 (s, 3H, 12-Me), 3.40 (s, 3H, 2-Me), 2.99 (s, 3H, 7-Me), 2.67-2.22 (m, 4H, 17a+17b-H), 1.97 (s, 3H, 8b-Me), 0.35 (each br s, NH).

Solid-phase binding assay for galectins. Binding between the carbohydrate-conjugated chlorins [in 0.1% (v/v) aqueous dimethyl sulfoxide (DMSO)] and the galectins [0.25-2 μg/100 μL in PBS (pH 7.4; Invitrogen, Carlsbad, USA)] was determined using a solid-phase assay in microtiter plate wells (SPL, Bucheon, Korea). Galectin-3 and galectin-8 were purchased from PeproTech Asia (Rehovot, Israel) and R&D Systems (Minneapolis, MN, USA), respectively.

The binding of galectins to carbohydrate-conjugated chlorins was detected using rabbit anti-galectin-3 antibody (Chemicon, Billerica, MA, USA) or rabbit anti-galectin-8 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-rabbit IgG peroxidase (Sigma-Aldrich Corp.). After the incubation of the binding conjugates with a 2,2-azino-bis (ethylbenzthiazoline-6-sulfonic acid) liquid substrate system (Sigma-Aldrich Corp.), the color was monitored at 405 nm using a Synergy HT multi-detection reader (Biotek, Winooski, VT, USA) at the Biohealth Products Research Center (BPRC) at Inje University (Gimhae, Korea). Individual experimental series were performed in duplicate, and experiments were carried out independently more than three times.

SDS-polyacrylamide gel electrophoresis. All chemicals, gels, and sample buffer for electrophoresis were purchased from Invitrogen. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on NuPAGE Novex 4-12% Bis-Tris gels according to the manufacturer's instructions in the XCell SureLock Mini-Cell Electrophoresis System (Invitrogen). Ten microliters of carbohydrate-conjugated chlorins was prepared using NuPAGE LDS sample buffer (4×). SeeBlue Plus2 pre-stained standard was used as a marker for SDS-PAGE. The gel was stained with SimpleBlue SafeStain, destained with deionized water, and dried according to the manufacturer's instructions (Invitrogen).

Protein determination. All chemical reagents were purchased from Sigma-Aldrich Corp. The amount of galectin bound to carbohydrate-conjugated chlorin compounds was determined in 96-well flat-bottom plates using the Bradford reagent with BSA as a standard. Bradford reactions contained 250 μL of Bradford reagent and 5 μL of protein standard solution or sample (0.25-1.1 μg/μL). After 15 min of incubation, the absorbance at 595 nm was measured using the Synergy HT multi-detection reader.

Fluorescence spectroscopy. Carbohydrate-conjugated chlorins at 0.5 μg/100 μL were reacted with galectins at various concentrations (twofold serial dilutions from 0.75 μg/100 μL to 0.062 μg/100 μL). Fluorescence was then measured using a Luminescence Spectrometer LS-50B (Perkin-Elmer Inc., Waltham, MA, USA) at the Central Laboratory of Gyeongsang National University (Jinju, Korea). Solutions prepared without galectins were used as controls. Additionally, the fluorescence emission intensity of samples prepared at 1/10 and 1/100 dilutions was determined under the same experimental conditions.

Results and Discussion

Synthesis of carbohydrate-conjugated chlorins. Among various solubilizing substitutions, carbohydrates have attracted the most attention for the synthesis of various photosensitizers. In addition to providing the molecule with polar hydroxy groups, the carbohydrate moiety might also direct the conjugate to a cell-surface target through specific binding.[18] In recent years, various carbohydrate derivatives of chlorin-based compounds have been synthesized for diagnostic and therapeutic investigations of various cancers.[18] We synthesized β-D-(+)-galactose-conjugated chlorin (Scheme 1) to bind specifically to galectins. The starting material methyl pheophorbide-a (compound **1**) was reacted with HBr in acetic acid at 0 °C to yield brominated chlorin (compound **2**). The synthesis of β-D-(+)-galactose-conjugated chlorin (compound **3**) was achieved by the introduction of D-(+)-galactose and anhydrous K_2CO_3 to **2**. The final product **3** was produced at 36% yield, and its chlorin UV-vis absorbance occurred at 664.6 nm.[22]

To evaluate whether â-lactose enhanced galectin binding, we also synthesized pyropheophorbide-a β-lactose ester (compound **6**), as illustrated in Scheme 2. Methyl pyropheophorbide-a (compound **4**), prepared from methyl pheophorbide-a **1** via collidin treatment was dissolved in trifluoroacetic acid to yield pyropheophorbide-a (compound **5**). The β-lactose-conjugated chlorin (compound **6**) was synthesized by the addition of β-lactose and dicyclohexyl carbodiimide to **5** and removal of the solvent. The final product **6** was produced at 22% yield, and its chlorin UV-vis absorbance occurred at 665.7 nm.[22]

Binding of galectins to carbohydrate-conjugated chlorins. From our preliminary studies of the binding of D-(+)-galactose to galectins at various concentrations (0.25-2 μg/100 μL), we found that the absorbance intensity at 405 nm for the binding was approximately 0.5 for galectin-3 and 0.9 for galectin-8. For β-lactose binding, the absorbance intensity at 405 nm was approximately 1.1 for galectin-3 and 0.9 for galectin-8. Also, the fraction of galectins bound to carbohydrates increased during up to 30 min of incubation time.

Solid-phase assays were used to test for the binding of galectin-3 and galectin-8 to the carbohydrate-conjugated chlorin derivatives. There were similar patterns for the optimal detectable concentrations for galectin binding with

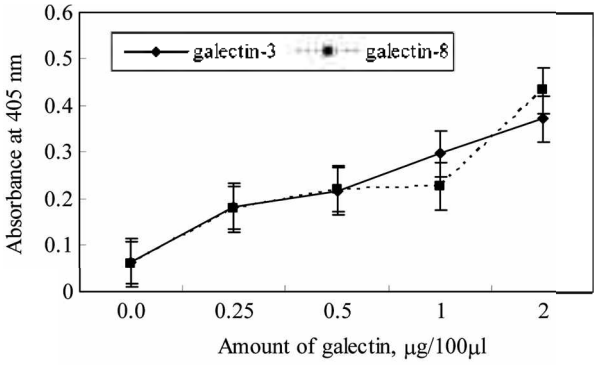


Figure 1. Binding of β-D-(+)-galactose-conjugated chlorin **3** to galectin-3 and galectin-8. Conjugated chlorin **3** was prepared at 1 mg/100 mL for optimal detectable binding, as shown in preliminary experiments.

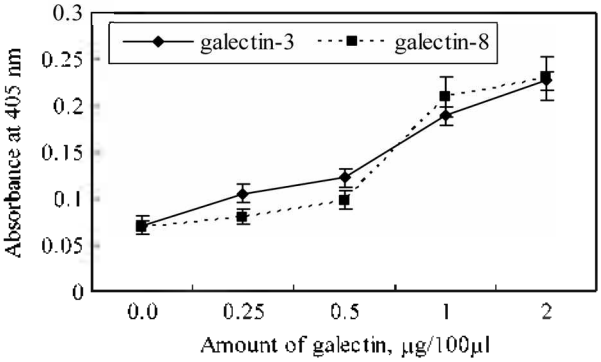


Figure 2. Binding of β-lactose-conjugated chlorin **6** to galectin-3 and galectin-8. Conjugated chlorin **6** was prepared at 1 μg/100 μL for optimal detectable binding, as shown in preliminary experiments.

D-(+)-galactose-conjugated chlorin **3** (Figure 1) and with β-lactose-conjugated chlorin **6** (Figure 2). Variation in the concentration of galectins had a significant effect on binding. The optimal concentrations of galectins for detection were between 0.5 and 1 μg/100 μL; the fraction of the detectable UV-visible absorbance at 405 nm was approximately 0.2-0.3 for conjugated chlorin **3** and 0.1-0.2 for conjugated chlorin **6**. The binding patterns of galectin-3 and galectin-8 were not markedly different, but their binding affinities were different. ELISA was suitable for the examination of the binding of galectin-3 and galectin-8 to both β-D-(+)-galactose-conjugated chlorin **3** and β-lactose-conjugated chlorin **6**.

Analysis of galectins. To verify that the conjugated chlorin-bound galectins retained their activity, we carried out SDS-PAGE. We then monitored the extent of galectin binding in bound fractions of the adsorbed conjugates and compared the relative levels of galectins bound to conjugates **3** and **6** (Figure 3). The molecular weights of bound galectin-3 and galectin-8 associated with conjugates **3** and **6** were 26 and 36 kDa, respectively. Thus, no structural damage to galectin-3 or galectin-8 occurred in the binding studies, and the galectins retained their activity.

Galectin binding efficiencies were determined using a

Figure 3. Galectin-3 and galectin-8 bound to β-D-(+)-galactose-conjugated chlorin **3** and β-lactose-conjugated chlorin **6**. Lane 1: standard markers, Lane 2: conjugate **3** bound with galectin-3 (0.5 μg/100 μL), Lane 3: conjugate **3** bound with galectin-3 (0.125 μg/100 μL), Lane 4: conjugate **3** bound with galectin-8, Lane 5: conjugate **6** bound with galectin-8.

Table 1. Efficiency of galectin binding to conjugates **3** and **6**

Conjugate	Galectin	Observation (μg/well)	Concentration		Bound fraction (%)
			Initial (μg/μL)	Bound (μg/μL)	
3	Galectin-3	4.81	1	0.962	96.2
3	Galectin-8	4.10	1	0.820	82.0
6	Galectin-3	4.41	1	0.882	88.2
6	Galectin-8	4.18	1	0.836	83.6

Bradford assay (Table 1). A larger fraction of galectin-3 than of galectin-8 bound to conjugates **3** and **6**. The highest binding fraction (96.2%) occurred for galectin-3 binding to β-D-(+)-galactose conjugate **3**. Overall, the galectins bound with at least 82% efficiency to the conjugates tested.

Fluorescence detection studies. Fluorescence measurements were taken using a Perkin-Elmer LS50B fluorometer with a 10-nm excitation/emission slit and 1.00 of integration time. For β-D-(+)-galactose-conjugated chlorin **3**, the excitation and emission wavelengths were 408 and 659 nm, respectively. For β-lactose-conjugated chlorin **6**, the excitation and emission wavelengths were 410 and 471 nm, respectively.

The intensity of fluorescence emission at 659 nm (the typical maximum for galectin-bound β-D-(+)-galactose-conjugated chlorin **3**) was less for galectin-bound conjugate **3** than for non-galectin-bound conjugate **3** (Figure 4). At galectin-3 and galectin-8 concentrations of 0.75 μg/100 μL, the fluorescence intensity was decreased by 30 and 34%, respectively, relative to that of non-galectin-bound conjugate **3**. When conjugate **3** was at 0.5 μg/100 μL, the optimal concentrations of galectin-3 and galectin-8 were 0.25-0.75 μg/100 μL and 0.5-0.75 μg/100 μL, respectively.

The intensity of fluorescence emission at 471 nm (the typical maximum for galectin-bound β-lactose-conjugated chlorin **6**) was less for galectin-bound conjugate **6** than for non-galectin-bound conjugate **6** (Figure 5). At galectin-3 and galectin-8 concentrations of 0.75 and 0.5 μg/100 μL, respectively, the fluorescence intensity was decreased by 22

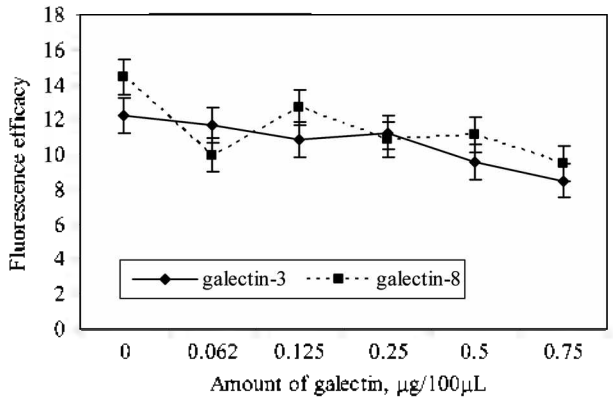


Figure 4. Fluorescence of galectin-bound β-D-(+)-galactose-conjugated chlorin **3** at various concentrations of galectin-3 and galectin-8. Conjugate **3** was prepared at 0.5 mg/100 mL for optimal binding.

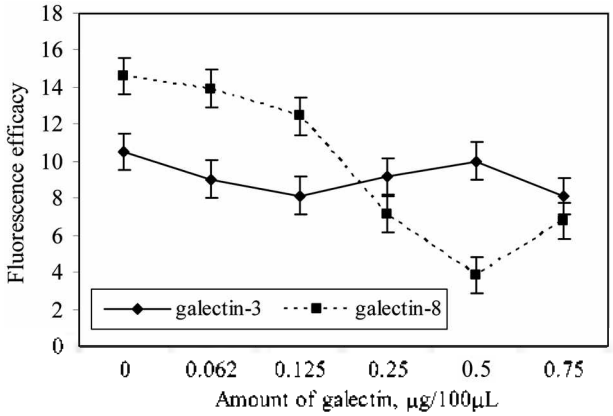


Figure 5. Fluorescence of galectin-bound β-lactose-conjugated chlorin **6** at various concentrations of galectin-3 and galectin-8. Conjugate **6** was prepared at 0.5 μg/100 μL for optimal binding.

and 70%, respectively, relative to that of non-galectin-bound conjugate **6**. When conjugate **6** was at 0.5 μg/100 μL, the optimal concentrations of galectin-3 and galectin-8 were 0.5-0.75 μg/100 μL and 0.062-0.5 μg/100 μL, respectively. Thus, conjugate **3** is more suitable for the detection of galectin-3, and conjugate **6** is more suitable for the detection of galectin-8.

We also investigated the intensity of fluorescence emission at 1/10 and 1/100 dilutions not only for conjugate **3** and **6**, but also for the galectins. The preliminary results showed that only the 1/10 dilution of conjugate **3** that was reacted with galectin-3 had no significant effect on fluorescence efficacy (Figures 4 and 5). This phenomenon suggests that β-D-(+)-galactose-conjugated chlorin **3** can be used as a fluorescence nanobiosensor for the galectin-3 cancer marker in the detection range of 0.05-0.075 μg/100 μL (500–750 ng/mL).

From the results of the fluorescence detection studies, we assume that electrons are transferred from the highly electron-dense carbohydrate-conjugated chlorins **3** and **6** to the bound galectin when the components are at acceptable levels. The change in electron density upon the binding of conjugates to galectins results in a change in fluorescence emission intensity. Thus, β-D-(+)-galactose-conjugated chlorin **3**, with high affinity for galectin-3, might be a very efficient detection tool for selective binding to the galectin-3 biomarker on the surface of cancer cells.

Acknowledgments. This research was supported by a fund of the Regional Industrial Technology Development Programs (No. 2006-70000403) of the Ministry of Commerce, Industry, and Energy, Republic of Korea.

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