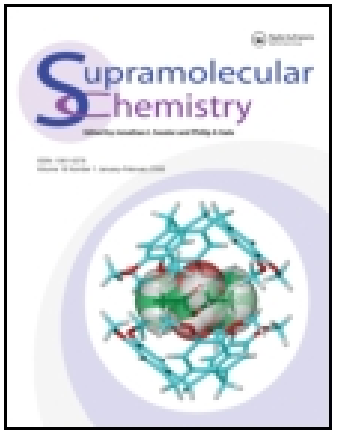

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Two different types of artificial glycosylated haemins (glycohaemins), in which a monosaccharide (galactose) or a disaccharide (lactobionic acid, 4-*O*-β-galactopyranosyl-D-gluconic acid) was introduced at the terminals of the two haem-propionate side chains, were synthesised to serve as a designed interface on the myoglobin surface. These glycohaemins were successfully inserted into apomyoglobin to yield an artificial glycoprotein by the conventional method. The interprotein interaction between the reconstituted myoglobin and peanut agglutinin lectin (PNA), a β-galactose-recognising protein, was confirmed by two different assay systems, i.e. a fluorometric assay using the fluorescein isothiocyanate-labelled lectin and an ELISA-like assay using the peroxidase-labelled lectin. The results revealed that each myoglobin reconstituted with the glycohaemin makes a complex with PNA, in which the glycoprotein with the disaccharides showed a higher binding affinity with the lectin compared to the glycoprotein with the monosaccharides, suggesting that the binding property clearly depends on the deposited carbohydrate surface on the myoglobin.

Keywords: protein–protein complex; haemoprotein modification; haem-substitution; artificial glycoprotein

Introduction

Biological systems have created sophisticated supramolecular assemblies of biomolecules, including proteins, nucleic acids, lipids and sugars, which enable them to integrate their various functions. To mimic and/or modify the naturally occurring supramolecular assemblies using an artificial methodology is one of the current interests from the view point of constructing nanobiomaterials (*1–5*). Especially, a cluster of carbohydrate molecules serves as a crucial recognition interface mediating protein–protein or protein–membrane interactions, which triggers cellular communications, such as cell adhesion, fertilisation, bacterial/viral infection and cancer metastasis (*6–8*). Therefore, carbohydrate molecules are attractive candidates for an interface displayed on a protein to construct an artificial supramolecular architecture.

During the past decade, many efforts have been devoted to developing at least three strategies producing artificially glycosylated proteins as a surrogate for post-translational modification (*9–11*): (i) preparation of a small glycoprotein by solid phase peptide synthesis using a glycosylated amino acid, (ii) introduction of carbohydrate moieties into a terminal of the residue of reactive amino acids such as Lys, Cys or Asp on the protein surface and (iii) expression of proteins using a genetically coded unnatural RNA with an amino acid having carbohydrate derivatives. These methods involve modification in order to form the carbohydrate-linked amino acid residues. In contrast, we have recently proposed a new method which is an introduction of carbohydrate moieties into a peripheral side chain of an enzyme cofactor (*12*). For example, several haemoproteins will be suitable for introducing a carbohydrate cluster onto the protein surface because it is well known that protohaem IX in myoglobin is readily replaced by an artificial haemin having a functional group at the haem-propionate side chains (*13–15*). In fact, we have already reported that the negatively charged myoglobin reconstituted with the artificially created haemin with a carboxylated cluster bound to the two haem-propionate side chains serves as a complex with the positively charged cytochrome *c* (*16*, *17*).

In our previous paper, we reported the preparation of myoglobin modified with tetradentate galactose units which has a sufficient binding affinity with peanut agglutinin lectin (PNA), a β-galactose binding protein, determined by a qualitative binding analysis (*12*). PNA is known to recognise the monosaccharide moiety and more strongly bind to the disaccharide; the interaction between the carbohydrate moiety and lectin depends on the structures and glycosylation pattern of the carbohydrates (*18*). To investigate the expandability of our reconstitutional strategy in constructing artificial glycoproteins, we now describe the preparation of myoglobins reconstituted

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with glycosylated haemins (glycohaemins) containing a monosaccharide unit, galactose (Gal) and a disaccharide unit, lactobionic acid (GlcGal) (Figure 1), as well as their sufficient binding abilities with the target lectin.

Experimental

Instruments

^{1}H NMR spectra were recorded on a Bruker AVANCE 400 (400 MHz) and/or JEOL JNM-EX 270 (270 MHz) spectrometers. The ^{1}H NMR chemical shifts were reported in ppm relative to the residual solvent resonances. Mass analysis was carried out using a time-of-flight mass spectrometer (TOF MS) equipped with electrospray ionisation on an Applied Biosystems Mariner API-TOF Workstation. UV–vis spectra were recorded using a Shimadzu UV-2550 double-beam spectrometer. Gel permeation chromatography (GPC) was performed on a LC-908 recycling preparative HPLC system (Japan Analytical Industry Co., Ltd, Tokyo, Japan) connected in series with JAIGEL-1H, JAIGEL-2H and JAIGEL-3H columns (Japan Analytical Industry Co., Ltd) using $CHCl_3$ as an eluent. Preparative high-performance liquid chromatography (HPLC) was performed by a Shimadzu SCL 10 Avp HPLC system. Purification of the reconstituted myoglobins (rMbs) was performed by an Amersham Bioscience $ÄKTA_{FPLC}$ system equipped with a Superdex 75 column and a fraction collector Frac-920 at 4°C. Optical absorptions for the microplate assays were determined on a Synergy HT SIAFR-4 (BIO-TEK Instruments, Inc., Winooski, VT, USA) at 470 nm. Fluorescent spectra were recorded using HITACHI F-4500. Distilled water was demineralised by a Barnstead NANOpure DIamond^{TM} apparatures.

Chemicals

All reagents and chemicals were obtained from commercial sources and used without further purification unless otherwise noted. Glycohaemins **1** and **2** were synthesised by the methods described below. Native horse heart myoglobin (nMb) was purchased from Sigma Co., Ltd (St Louis, MO, USA; M-1882). Bovine serum albumin (BSA) and horseradish peroxidase-labelled peanut agglutinin lectin (HRP-PNA) (L7759) were purchased from Sigma Co., Ltd. Fluorescein isothiocyanate-labelled peanut agglutinin lectin (FITC-PNA) was purchased from Seikagaku Corporation (Tokyo, Japan).

Synthetic procedures

General

Protoporphyrin IX having four carboxylic acid groups (**3**) was synthesised according to our previous paper (*12*). 2-(*p*-Nitrobenzyloxycarbonylamino)ethylamine (*19*) and 2-(benzyloxycarbonylamino)ethylamine (*20*) were prepared according to the procedures reported in the literature.

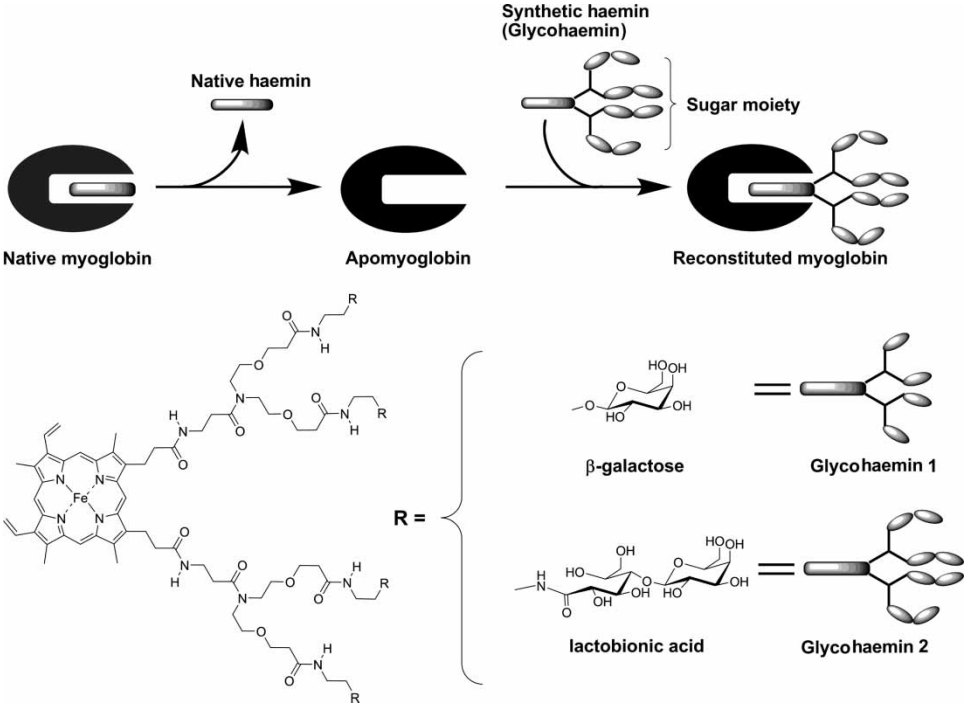


Figure 1. Schematic representation of the reconstitution strategy of myoglobin with synthetic haemin (glycohaemin).

*Synthesis of 2-(p-nitrobenzyloxycarbonylamino)ethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (**4**)*

To a solution of 2-(*p*-nitrobenzyloxycarbonylamino)ethylamine (2.7 g, 11.2 mmol) and 1,2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (3.9 g, 10 mmol) in CH_2Cl_2 (90 ml) was added dropwise to a solution of boron trifluoride diethyletherate (9.0 ml, 71 mmol) at 0°C. The reaction mixture was stirred at 0°C for 30 min, followed by at room temperature for 12 h. The solution was neutralised by $NaHCO_3$ aq., and the organic phase was washed with H_2O and brine. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by silica gel chromatography (hexane/AcOEt = 1/4) to give **4** (1.7 g, 30%). ^{1}H NMR (270 MHz, $CDCl_3$) δ (ppm) 8.21 (2H, d, J = 8.9 Hz), 7.50 (2H, d, J = 8.9 Hz), 5.38 (1H, d, J = 2.4 Hz), 5.26–5.14 (4H, m), 5.00 (1H, dd, J = 3.5, 10.7 Hz), 4.46 (1H, d, J = 7.8 Hz), 4.19–4.06 (2H, m), 3.92–3.84 (2H, m), 3.72–3.65 (1H, m), 3.44–3.37 (2H, m), 2.17–1.85 (12H, m).

*Synthesis of 2-aminoethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (**5**)*

To a solution of **4** (1.7 g, 3.0 mmol) in MeOH (30 ml) was added 10% Pd/C (170 mg). After addition of AcOH (0.36 g, 6.0 mmol), the reaction mixture was stirred at room temperature under H_2 atmosphere for over 2 h. The Pd/C was removed by filtration, and then the filtrate was evaporated to dryness. The residue was lyophilised for 2 days to remove *p*-toluidine. The resulting residue was dissolved in CH_2Cl_2 (20 ml), and *p*-toluenesulphonic acid monohydrate (570 mg, 3.0 mmol) was added. The solution was stirred at room temperature for 1.5 h. The solvent was evaporated and co-evaporated with toluene three times to give precipitate **5** as a tosylate salt (418 mg, 25%). ^{1}H NMR (270 MHz, $CDCl_3$) δ (ppm) 7.71 (2H, d, J = 8.1 Hz), 7.21 (2H, d, J = 8.1 Hz), 5.34 (1H, d, J = 2.7 Hz), 5.15–5.08 (1H, m), 4.94 (1H, dd, J = 3.2, 10.5 Hz), 4.48 (1H, d, J = 8.1 Hz), 4.19–4.07 (2H, m), 4.04–3.99 (2H, m), 3.88–3.83 (1H, m), 3.30–3.15 (2H, m), 2.29–1.90 (15H, m).

*Synthesis of porphyrin having peracetylated galactoside (**8**)*

To a solution of **5** (418 mg, 0.74 mmol), **3** (126 mg, 0.17 mmol) and 1-hydroxybenzotriazole monohydrate (114 mg, 0.74 mmol) in dry DMF (6 ml) was slowly added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (335 mg, 1.75 mmol) at 0°C. The reaction mixture was stirred at 0°C for 1 h, followed by at room temperature for 12 h. The solution was diluted with $CHCl_3$ and the organic phase was washed with water, 5% citric acid, sat. $NaHCO_3$ aq. and brine. The organic phase was dried over Na_2SO_4 and evaporated to give a purple solid. The solid was subjected to silica gel chromatography ($CHCl_3$/MeOH = 9/1) and recycle GPC to afford purple solid **8** (124 mg, 27%). ESI-TOF MS (positive mode) *m/z* 887.82 $(M+3H)^{3+}$, calcd for $(C_{124}H_{173}N_{12}O_{52})^{3+}$ 887.37; UV–vis ($CHCl_3$) λ_{max} (nm) 403, 504, 538, 574, 628 and 664.

*Synthesis of glycohaemin **1***

To a solution of **8** (124 mg, 46 μmol) and suspended $NaHCO_3$ (386 mg, 0.46 mmol) in 6 ml of N_2-purged $CHCl_3$/MeCN (1/1, v/v) was added ferrous chloride tetrahydrate (92 mg, 0.46 mmol). The reaction mixture was stirred at 50°C for 2 h and cooled to room temperature. The mixture was exposed to air and diluted with $CHCl_3$ and washed with water, sat. $NaHCO_3$ aq. and brine. The organic phase was dried over Na_2SO_4 and evaporated to dryness. Half of the obtained residue was dissolved in MeOH (5 ml) and NaOMe (5.5 mg, 100 μmol) was added. The mixture was stirred at room temperature for 4 h and 100 μl of 0.1 M HCl aq. was added. After the solvent was evaporated, the resulting residue was subjected to LH-20 gel filtration with elution of water to give glycohaemin **1** (31 mg, 64%). ESI-TOF MS (positive mode) *m/z* 1021.34 $(M+H^+-Cl)^{2+}$ calcd for $(C_{92}H_{137}FeN_{12}O_{36})^{2+}$1020.93; UV–vis (100 mM posphate buffer) λ_{max} (nm) 397 (sh) and 596.

*Synthesis of 2-(benzyloxycarbonylamino)ethyl (2,3,5,6,2′,3′,4′,6′-octa-O-acetyl)lactobionamide (**6**)*

To a suspension of lactobionic acid (7.5 g, 21 mmol) in MeOH (200 ml) was added 2-(benzyloxycarbonylamino)ethylamine (4.1 g, 21 mmol). The reaction mixture was stirred at 75°C for 6 h. The solvent was evaporated to give a semi-solid compound. The obtained residue was dissolved in pyridine (270 ml) and cooled with ice bath. Acetic anhydride (170 ml) was added dropwise at 0°C and the reaction mixture was stirred at room temperature for 12 h. The reaction was quenched by addition of MeOH (170 ml) at 0°C and the solvent was removed by evaporation. The residue was dissolved in AcOEt and the organic phase was washed with water, 0.1 M HCl aq., sat. $NaHCO_3$ aq. and brine. The organic phase was dried over Na_2SO_4 and evaporated to give crude **9**. The residue was purified by silica gel chromatography (hexane/AcOEt = 1/3–AcOEt only) to give white solid **6** (7.8 g, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.33–7.24 (5H, m), 6.80 (1H, br), 5.61 (1H, d, J = 5.9 Hz), 5.49 (1H, dd, J = 3.5, 6.0 Hz), 5.35 (1H, d, J = 3.0 Hz), 5.30 (1H, br), 5.16 (1H, dd, J = 8.1, 10.6 Hz), 5.10–5.02 (3H, m), 4.98 (1H, dd, J = 3.5, 10.4 Hz), 4.62 (1H, d, J = 8.1 Hz), 4.52 (1H, dd, J = 2.7, 12.4 Hz), 4.38 (1H, br), 4.18–4.14 (1H, m), 4.04–3.98 (2H, m), 3.87 (1H, t, J = 6.6 Hz), 3.45–3.20 (4H, m), 2.17 (3H, s), 2.12 (3H, s), 2.05 (6H, s), 2.03 (3H, s), 2.00 (6H, s), 1.96 (3H, s).

*Synthesis of 2-aminoethyl (2,3,5,6,2′,3′,4′,6′-octa-O-acetyl)lactobionamide (**7**)*

To a solution of **6** (3.4 g, 3.9 mmol) in AcOH (40 ml) was added 10% Pd/C (200 mg). The reaction mixture was stirred at room temperature under H_2 atmosphere over 12 h. The Pd/C was removed by filtration, and then the filtrate was evaporated to dryness. The resulting mixture was dissolved in CH_2Cl_2 (20 ml), and *p*-toluenesulphonic acid monohydrate (740 mg, 3.9 mmol) was added. The solution was stirred at room temperature for 1.5 h. The solvent was evaporated and co-evaporated with toluene three times to give white solid **9** as a tosylate salt (3.4 g, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.03 (1H, br), 7.69 (2H, d, $J = 7.8$ Hz), 7.51 (3H, br), 7.17 (2H, d, $J = 7.8$ Hz), 5.50 (1H, br), 5.44 (1H, br), 5.34 (1H, d, $J = 3.2$ Hz), 5.11–5.06 (2H, m), 4.97 (1H, dd, $J = 3.4$, 10.5 Hz), 4.57 (1H, d, $J = 7.8$ Hz), 4.53 (1H, br), 4.28 (1H, br), 4.16 (1H, br), 4.08–3.96 (2H, m), 3.85 (1H, t, $J = 6.5$ Hz), 3.48 (2H, br), 3.16 (2H, br), 2.11–1.94 (27H, m).

*Synthesis of porphyrin having peracetylated lactobionamide (**9**)*

To a solution of **3** (200 mg, 0.17 mmol), **9** (2.5 g, 2.72 mmol) and triethylamine (2 ml, 14 mmol) in DMF (7 ml) was added 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (1.75 g, 6.3 mmol). The reaction mixture was stirred at room temperature for 12 h. The solvent was evaporated and the residue was diluted with CH_2Cl_2. The organic phase was washed with water, 0.1 M HCl aq., sat. $NaHCO_3$ aq. and brine. The organic phase was dried over Na_2SO_4 and evaporated to give crude **9**. The residue was purified by silica gel chromatography ($CHCl_3$/MeOH = 9/1–6/1) to give a purple solid. The solid was further purified by recycle GPC to give **9** (200 mg, 32%). ESI-TOF MS (positive mode) *m/z* $(M+3H^+)^{3+}$ 1348.74 calcd for $(C_{180}H_{249}N_{16}O_{88})^{3+}$ 1347.52; UV–vis ($CHCl_3$) λ_{max} (nm) 408, 505, 541, 574, 630 and 668.

*Synthesis of glycohaemin **2***

To a solution of **9** (40 mg, 9.9 μmol) and suspended $NaHCO_3$ (15 mg, 0.18 mmol) in 4 ml of N_2-purged $CHCl_3$/MeCN (1/1, v/v) was added ferrous chloride hydrate (40 mg, 0.20 mmol). The reaction mixture was stirred at 70°C for 2 h and cooled to room temperature. The mixture was exposed to air and diluted with $CHCl_3$ and washed with 1 M HCl aq. and brine. The organic phase was dried over Na_2SO_4 and evaporated. The obtained residue was dissolved in 2 ml of MeOH/H_2O (1/1, v/v) and NaOMe (5.5 mg, 100 μmol) was added. After the reaction mixture was stirred at room temperature for 5 h, amberlite IRC-50 was added. The IRC-50 was removed by filtration and the solvent was evaporated. The resulting residue was purified by reverse phase HPLC (YMC-pack C18, 250 × 20 mm) to give glycohaemin **2** (10 mg, 36%). The HPLC condition was as follows: CH_3CN/H_2O (both containing 0.1% TFA) 10/90–50/50 (linear gradient over 50 min), flow rate = 5.0 ml/min, detected by UV (400 nm). ESI-TOF MS (positive mode) *m/z* $(M+H^+-Cl)^{2+}$ 1375.44 calcd for $(C_{116}H_{181}FeN_{16}O_{56})^{2+}$ 1375.06; UV–vis (H_2O) λ_{max} (nm) 358 (sh), 397 and 598.

Reconstitution of myoglobin with glycohaemin

The nMb was dissolved in 10 mM phosphate buffer (pH 6.0) and purified by cation exchange chromatography using a CM-52 cellulose column as previously described (*13*). The horse heart apomyoglobin (apoMb) was prepared from purified nMb by Teale's acid-2-butanone method (*21*), lyophilised and then stored at −80°C before use. To a solution of apoMb in 100 mM phosphate buffer (pH 7.0) was added dropwise an aqueous solution of 1.2 equivalent of glycohaemin **1** with gentle shaking, followed by overnight incubation at 4°C. The rMb with glycohaemin **1**, rMb–Gal, was purified from the residual glycohaemin **1** using a Superdex 75 column (10 mM phosphate buffer containing 154 mM sodium chloride as an eluent) in the FPLC system. The rMb with glycohaemin **2**, rMb–GlcGal, was obtained according to a similar procedure. The approximate yield for inserting the glycohaemin into apoMb was 40–50%.

Fluorescent quenching assay

The fluorescence spectra were measured in a quartz microcell at 25°C and recorded from 500 to 600 nm with excitation at 490 nm. Aliquots (150 μM, 2 μl) of the stock solution of nMb, rMb–Gal or rMb–GlcGal were added to a solution of FITC-PNA (43 μM, 60 μl). A 10 mM Tris–HCl buffer (pH 7.0) containing 10 mM $MnCl_2$ and $CaCl_2$ was used. The changes in the relative FITC intensity at the fluorescence maxima (520 nm) were plotted versus the myoglobin concentration with the Stern–Volmer analysis,

$$\frac{F_0}{F} = 1 + K_S[Q],$$

where F_0 and F are the fluorescence intensity in the absence and presence of the quencher (rMb–GlcGal, rMb–Gal and nMb), respectively. K_S represents the association constant and [*Q*] is the concentration of the quencher (*22*).

Microplate assay

The commercially available Nunc Maxisorp F96 microwell plates were used for this assay and the procedures are described below. The wells were coated with a solution of nMb, rMb–Gal or rMb–GlcGal (2 μM, 100 μl). The plate

was incubated overnight at room temperature to immobilise the protein on the well surface. The protein solution was removed, and the wells were washed three times with phosphate buffered saline (PBS; 250 μl), to remove any unbound proteins. The redundant well surface was blocked by the addition of 1% BSA in PBS (100 μl), with incubation for 1 h so as to avoid the non-specific binding of the PNAs to the well. The blocking solution was removed, and the wells were washed three times with PBS (250 μl). HRP-PNA (50 μg/ml, 100 μl) was added and the plate was incubated for 2 h. The protein solution was removed and the wells were washed three times with PBS (250 μl). To each well were added the substrate solution (guaiacol, 1 mM, 100 μl) and H_2O_2 (1 mM, 100 μl). The increases in the 470 nm absorbance, which corresponds to the oxidised product catalysed by HRP, were observed on a microplate reader.

Results and discussion

Preparation of glycohaemins

Two artificial haemins having tetradentate monosaccharide (glycohaemin **1**) or disaccharide (glycohaemin **2**) moieties were synthesised according to the synthetic route shown in Scheme 1. Galactose was coupled with 2-(*p*-nitrobenzyloxycarbonylamino)ethylamine and then the protecting group was removed by hydrogenation to obtain the galactose with the amino group, **5**. We also prepared the corresponding Z-protected galactose; however, the removal of the Z group was unsuccessful under the general hydrogenation conditions, whereas in the case of the synthesis of **6**, the removal of the Z group proceeded very smoothly to give the disaccharide analogue with the amino group, **7**. These sugar moieties were coupled to the modified protoporphyrin IX precursor **3** with bidentate carboxyl linkers at the terminal of both propionate chains, according to our previous method (*12*).

Iron insertion into the glycosylated protoporphyrins **8** and **10** was carried out under conventional conditions. The glycohaemins **1** and **2** were obtained by removal of the acetyl groups under the alkaline conditions, and purified by gel filtration and/or reverse phase HPLC techniques. The porphyrins and metalloporphyrins are generally known to be aggregated and insoluble because of their hydrophobic nature, while our glycohaemins with attached multiple carbohydrate moieties exhibit a high solubility in aqueous solutions at physiological pH (10 mM phosphate buffer, pH 7.0) in which the glycohaemins existed as the monomer and μ-oxo dimer indicated by the characteristic absorption at 605, 397 and 360 nm (sh).

Reconstitution of myoglobin with glycohaemin

The rMbs with glycohaemins were prepared by the conventional method. Upon the addition of the glycohaemin to the apoprotein, the Soret band of the glycohaemin shifted from 397 to 408 nm, suggesting that the modified haemin is incorporated into the myoglobin haem pocket and is coordinated with His93 to afford the five-coordinate Fe(III) species.

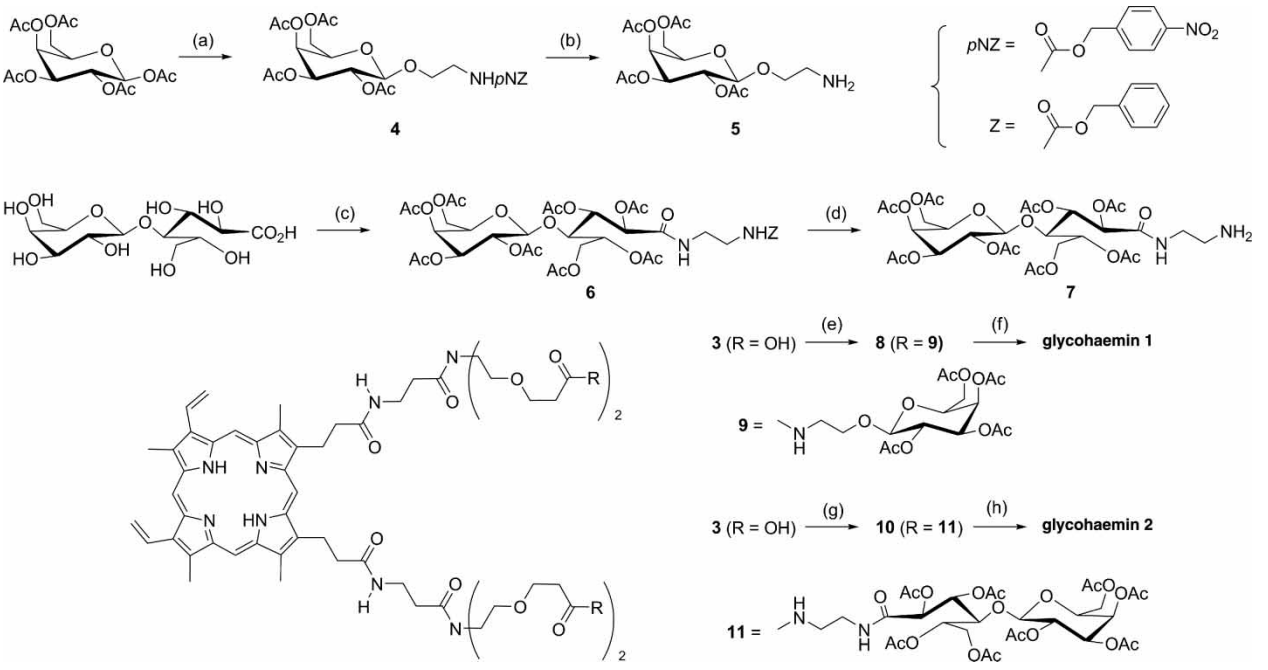
The rMbs were confirmed by UV–vis and ESI-TOF MS spectroscopic methods and size exclusion chromatographic analysis (SEC). The UV–vis absorption spectra of rMbs showed the characteristic Soret absorptions at 408 nm and the Q absorption at 506 and 633 nm similar to those of nMb, indicating that the glycohaemins were incorporated at the proper position with the same orientation in the haem pocket of the myoglobin (Figure 2). The ESI-TOF MS spectra of rMb with glycohaemin **1** (rMb–Gal) and rMb with glycohaemin **2** (rMb–GlcGal) exhibited the corresponding multiple charged ions of the holo- and/or apoproteins.[1] The SEC traces of nMb (17.5 kDa), rMb–Gal (19 kDa) and rMb–GlcGal (20 kDa) are in good agreement with the relative increase in their molecular size/weight because of the attached carbohydrate moiety anchored at the terminal of the two haem-propionate side chains (Figure 3).

Fluorescence quenching assay

To evaluate the interaction between the carbohydrate moieties attached on the myoglobin surface and PNA, we carried out fluorescence quenching assay using the FITC-PNA (*23–25*), in which the fluorescence can be quenched by the iron-containing haemin group when the FITC chromophore and the haemin are located within the acceptable distance. Thus, the decay of the FITC fluorescence intensity can serve as a useful probe to estimate the complexation between the PNA and the sugar moiety linked to the myoglobins.

The changes in the relative FITC intensity at the fluorescence maxima (520 nm) were plotted versus the myoglobin concentration as shown in Figure 4. In the case of the nMb, the FITC fluorescence intensity slightly decreased upon the addition of the myoglobin solution because of non-specific interactions between PNA and nMb. However, in the case of glycomyoglobins, rMb–Gal and rMb–GlcGal, more significant decays in the fluorescence intensity were observed with the increased concentration of the reconstituted proteins, suggesting specific binding between the attached carbohydrate groups on the protein surface and PNA. Furthermore, the results revealed that the terminal galactoside residue in the disaccharide-conjugated myoglobin, rMb–GlcGal, exhibits superior binding as compared with the monosaccharide-conjugated myoglobin, rMb–Gal.

The PNA forms homotetramer in which each domain has one substrate binding site. The four binding sites in the PNA are sufficiently separated by a distance of more than 50 Å. The maximum distance between the sugar moieties



Scheme 1. Syntheses of glycohaemins **1** and **2**. Reagents: (a) (*p*NZ)HNCH$_2$CH$_2$OH, $BF_3{\cdot}Et_2O$, CH_2Cl_2; (b) (i) H_2, 10% Pd/C, MeOH, AcOH; (ii) lyophilisation; (iii) *p*-toluenesulphonic acid monohydrate, CH_2Cl_2; (c) (i) ZHNCH$_2$CH$_2$NH$_2$, MeOH; (ii) Ac_2O, pyridine; (d) (i) H_2, 10% Pd/C, AcOH; (ii) *p*-toluenesulphonic acid monohydrate, CH_2Cl_2; (e) **5**, HOBt·H_2O, EDC·HCl, DMF; (f) (i) $FeCl_2$, $NaHCO_3$, $CHCl_3$/MeCN; (ii) NaOMe, MeOH; (g) **7**, DMT-MM, DMF; (h) (i) $FeCl_2$, $NaHCO_3$, $CHCl_3$/MeCN; (ii) NaOMe, MeOH/H_2O.

in the synthetic haemin is around 30 Å, and hence it seems rather difficult for one glycosylated Mb to occupy two of the four binding sites in a bidentate fashion at the same time. The Stern–Volmer plot showed a linear relationship under diluted condition, indicating that the binding site is independently occupied without any allosteric effect. Using the fluorescence quenching results, the association constant of the three Mbs with each domain of the PNA was determined as follows: rMb–GlcGal ($2.7 \times 10^4\,M^{-1}$), rMb–Gal ($1.3 \times 10^4\,M^{-1}$) and nMb ($5.3 \times 10^3\,M^{-1}$). In fact, it is known that the PNA binds the tumour-associated T-antigenic disaccharide, Galβ1, 3GalNAc, more strongly than the monosaccharide, galactose (*26*). It seems likely that the gluconic acid of lactobionic acid additionally supports the binding event by making hydrogen bonds with PNA. In our case, 'glycomyoglobin' clearly binds to PNA via specific carbohydrate–lectin interactions, which are dependent on the structure of the carbohydrate.

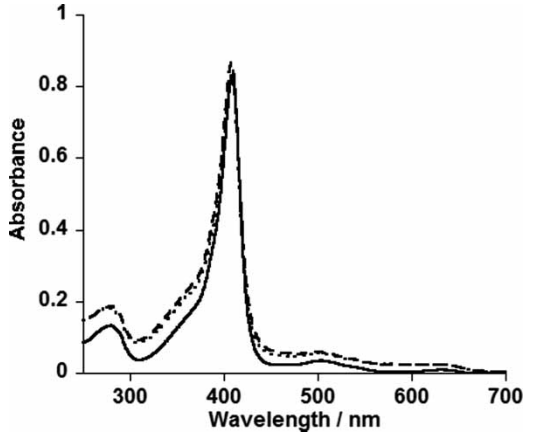


Figure 2. UV–vis spectra of rMb–GlcGal (dotted line), rMb–Gal (dashed line) and nMb (solid line) in 10 mM phosphate buffer at pH 7.0, 25°C.

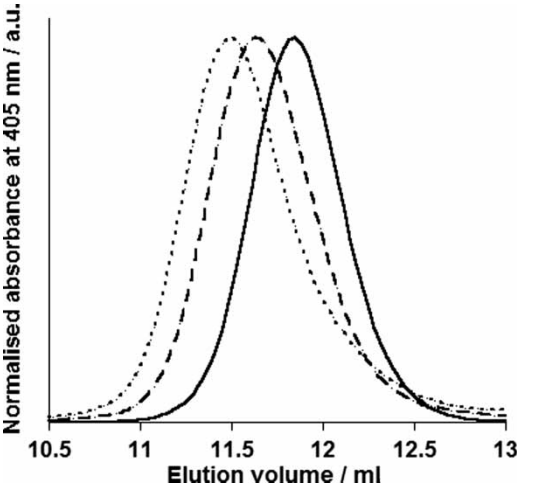


Figure 3. Size exclusion chromatographic profiles of rMb–GlcGal (20 kDa; dotted line), rMb–Gal (19 kDa; dashed line) and nMb (17.5 kDa; solid line).

Microplate assay

The above-mentioned results in the fluorescence quenching experiments showed that our glycomyoglobins have the potential to provide a useful binding interface for sugar-binding proteins, such as lectins, in a homogeneous solution. To demonstrate this strategy which is applicable for other varieties of sugar moieties, high-throughput screening methods would be desirable. Thus, we assessed the binding ability of the rMbs to PNA in heterogeneous circumstances using a microplate. The microplate assay is similar to the well-known ELISA (*11*, *27–30*), which has already been demonstrated as a useful methodology for high-throughput screening for biomolecular interactions, such as protein–protein, protein–DNA and protein–small molecule interactions. In our case, the reconstituted protein was adsorbed on a microwell and treated with the HRP-PNA conjugate as shown in Figure 5. Guaiacol, 2-methoxyphenol, was used as a substrate for the oxidation catalysed by HRP-PNA, because it is catalytically converted to the oxidised product which is determined by spectral changes at 470 nm (*31*). The binding fashion between PNA and glycomyoglobin was confirmed by a time-course study of the increased absorption of the oxidised guaiacol as shown in Figure 6. The profiles for rMb–Gal and rMb–GlcGal revealed a significant increase in the absorbance of the oxidised product. The native myoglobin is assumed to bind to PNA non-specifically, resulting in the slight increasing of the absorbance. The reconstituted proteins accelerate the guaiacol oxidation, suggesting that the introduction of the carbohydrate cluster on the protein surface acts as an interface for PNA. In particular, the faster guaiacol oxidation catalysed by HPR-PNA in the presence of rMb–GlcGal demonstrates a higher affinity of rMb–GlcGal for PNA compared to rMb–Gal. Furthermore, it was found that the addition of the excess amounts of galactose to the solution inhibited the guaiacol oxidation, supporting the fact that the PNA substrate binding sites selectively interact with the carbohydrate cluster deposited on the myoglobin surface. These results are in good agreement with the fluorescence quenching assay, suggesting that the rMb immobilised on the well surface provides the effective orientation of the carbohydrate units as a recognising interface. Thus, we found that the microplate assay is an appropriate method for assessing the binding potential of the carbohydrate

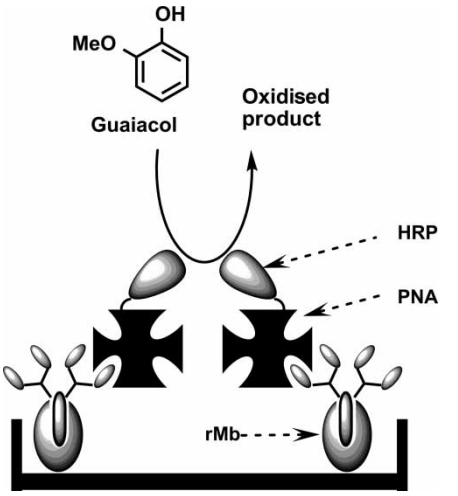


Figure 5. Schematic representation of the principle of the microplate assay technique.

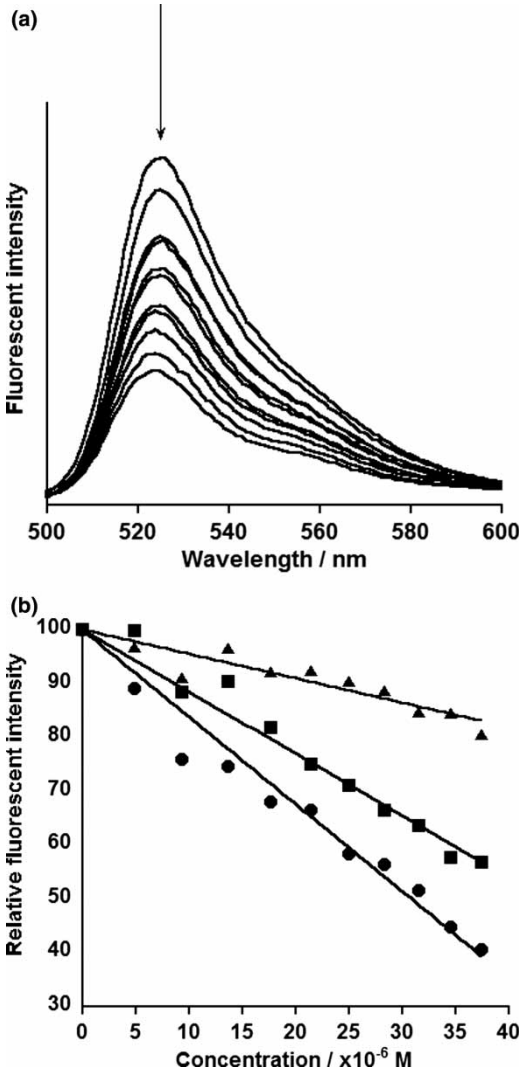


Figure 4. (a) Fluorescent spectral changes for FITC excited at 490 nm upon the addition of rMb–GlcGal in 10 mM Tris–HCl buffer (pH 7.0), $[CaCl_2] = [MnCl_2] = 10$ mM at 25°C. The concentration of protein is as follows: [rMb–GlcGal] = 0, 4.8, 9.4, 14, 18, 21, 25, 28, 32, 35 and 38 μM and [FITC-PNA] = 43, 42, 41, 39, 38, 37, 36, 35, 34, 33 and 32 μM. (b) Relative fluorescent intensities (520 nm) versus titrated concentration of myoglobins. rMb–GlcGal (filled circles), rMb–Gal (filled squares) and nMb (filled triangles).

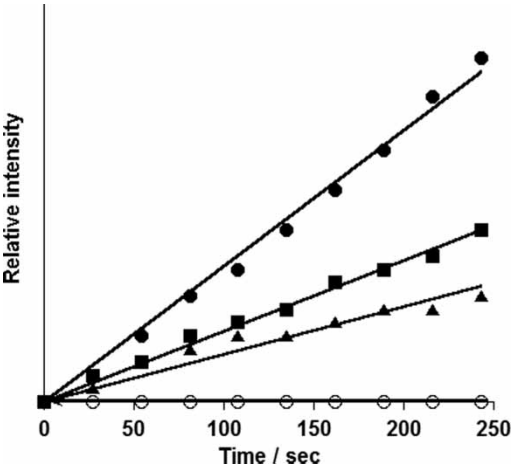


Figure 6. Time course profiles of relative absorption intensities of oxidised products (460 nm) catalysed by HRP-PNA in the presence of rMb–GlcGal (filled circles), rMb–Gal (filled squares) and nMb (filled triangles). The relative intensities obtained by rMb–GlcGal showed no changes without HRP-PNA (open circles); [nMb] = [rMb–GlcGal] = [rMb–Gal] = 2 μM and [HRP-PNA] = 50 mg/ml.

moieties as the interface to form the myoglobin–PNA complex and the dependence of the carbohydrate structure.

Summary

We synthesised artificially modified haemins having a monosaccharide unit, glycohaemin **1**, and a disaccharide unit, glycohaemin **2**. These glycohaemins were smoothly inserted into apoMb to give rMbs, which were well characterised by UV–vis and ESI-TOF MS spectroscopies and SEC analysis. The binding behaviour of rMb was evaluated by two independent assays, suggesting that the carbohydrate moieties attached on the protein surface have the function to form the supramolecular complex with the sugar-binding protein, lectin. Furthermore, the binding affinity of rMb for lectin depended on its attached sugar structure.

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Note

1. The apoprotein was formed under ionisation condition.

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