Hydrogen peroxide-triggered conversion of boronic acid-appended insulin into insulin and its application as a glucose-responsive insulin formulation

Hinako Kikuchi,[a] *Yuki Nakamura,*[a] *Chika Inoue,*[a] *Sayaka Nojiri,*[a] *Miho Koita,*[a] *Minori Kojima,*[a] *Hiroki Koyama,*[a] *Ryotaro Miki,*[a] *Toshinobu Seki,*[a] *Yuya Egawa**[a]

[a]Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan

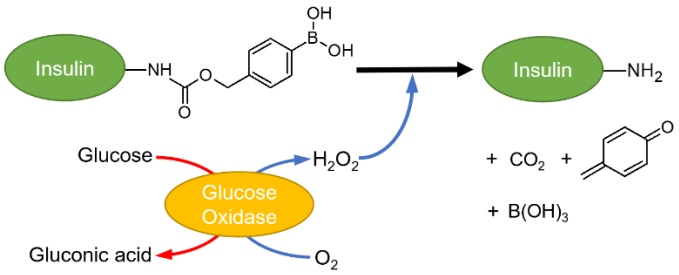
Corresponding Author

*Email: yegawa@josai.ac.jp; Tel: +81-49-271-7686; Fax: +81-49-271-7714

ORCID: 0000-0002-3317-9102

Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan

Graphical Abstract



Abstract

p-Boronophenylmethoxycarbonyl (BPmoc) is a protecting group for amines that is removable by treatment with hydrogen peroxide (H_2O_2). We prepared BPmoc-modified insulin (BPmoc-Ins) and subcutaneously injected the formulation into diabetic rats. The results demonstrated that BPmoc effectively sealed the blood glucose (Glc)-lowering effects of Ins. Conversely, co-injection of BPmoc-Ins and Glc oxidase (GOx) resulted in reduced blood Glc levels, indicating that Ins was generated from BPmoc-Ins through the following reactions: oxidation of endogenous Glc by GOx; production of H_2O_2 accompanied by Glc oxidation; and removal of BPmoc residues by H_2O_2. These results show the potential of BPmoc-Ins for a Glc-responsive Ins release system.

Keywords: insulin; glucose-responsive release; boronic acid; hydrogen peroxide; hypoglycemia

Introduction

In the field of organic chemistry, phenylboronic acid derivatives have been recognized as substrates

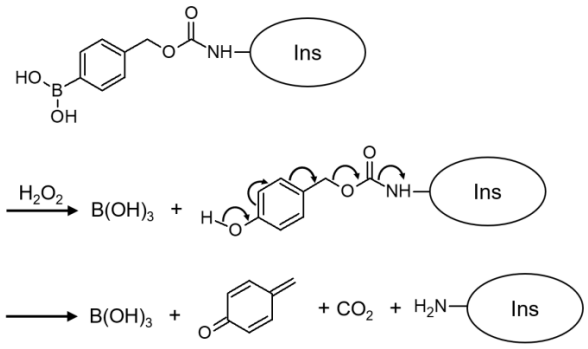
of Suzuki–Miyaura cross-coupling reactions.[1,2] In the field of supramolecular chemistry, these compounds have been widely used as sugar recognition motifs for sugar sensors because phenylboronic acid and sugar react to form a cyclic ester.[3–8] Additionally, phenylboronic acid derivatives have attracted attention for their response to hydrogen peroxide (H_2O_2).[9–12] H_2O_2 causes the replacement of a boronic acid group with a hydroxyl group. This reaction is used for many H_2O_2 sensors in which a boronic acid residue is appended to a chromophore or fluorophore. This type of reactions includes a special reaction in which a *p*-boronophenylmethoxycarbonyl (BPmoc) group reacts with H_2O_2, leading to its decomposition into boric acid, *p*-quinone methide, and CO_2[13–19] as well as the elimination of the BPmoc group from its attached site, as presented in Scheme 1.

Using the characteristic reactivity of BPmoc for H_2O_2, Ikeda *et al.* developed H_2O_2-responsive supramolecular hydrogels.[18] They appended BPmoc to phenylalanine dipeptide (BPmoc-FF) and confirmed the formation of supramolecular hydrogel through the self-assembly of BPmoc-FF *via* π-π interaction and hydrogen bonding. They inserted glucose oxidase (GOx) into the BPmoc-FF hydrogel to produce H_2O_2 through glucose (Glc) oxidation. When the BPmoc-FF hydrogel was immersed in a Glc solution, BPmoc was eliminated from BPmoc-FF by H_2O_2 produced *via* Glc oxidation, which results in gel-sol transformation through the destruction of intermolecular interactions forming the supramolecular structure of BPmoc-FF hydrogel. They also described the Glc-triggered release of insulin (Ins) labeled with a fluorophore from the BPmoc-FF hydrogel. This is a successful *in vitro* example of a Glc-responsive Ins release system that is expected to serve as an

ideal Ins formulation for diabetes treatment. Ins is the only blood Glc-lowering hormone, and it is used as a diabetes treatment. However, Ins treatment always carries a risk of hypoglycemia.[20] To reduce the risk of hypoglycemia, Glc-responsive Ins release systems have been extensively investigated for 30 years.[21–25] Nevertheless, such systems have not yet been applied practically, indicating the difficulty of developing such systems. In general approaches, drug carriers or supports for Glc-responsive Ins release systems simultaneously require several well-designed functions: loading of Ins; sensing of Glc; and Glc-dependent releasing of Ins, which would explain the difficulty of developing such systems.

Without developing complicated drug carriers or supports, we attempted a simple approach to develop a Glc-responsive Ins release system using the character of BPmoc. We directly attached BPmoc to Ins. At first glance, BPmoc-modified Ins (BPmoc-Ins) resembles previously reported prodrugs that are responsive to endogenous H_2O_2.[26–28] However, our strategy is unique in that we administered GOx with BPmoc-Ins as an injection without any drug carriers or supports. The co-injected GOx produced H_2O_2 by oxidizing endogenous Glc in the injected part. The produced H_2O_2 eliminated BPmoc from BPmoc-Ins, as illustrated in Scheme 1, and the generated Ins was expected to lower blood Glc levels. Thus, the combination of BPmoc-Ins and GOx has potential as a stimuli-sensitive Ins release system that responds to endogenous Glc. In this communication, we discussed the details of this approach.

Scheme 1. H_2O_2-triggered conversion of BPmoc-Ins into Ins.



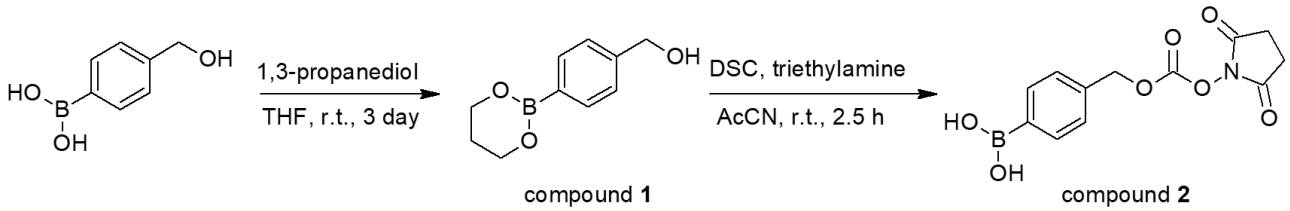
Results and Discussion

Synthesis

As presented in Scheme 2, 4-(hydroxymethyl)phenylboronic acid was used as a starting compound to prepare compound **2**, which is an active ester of BPmoc. First, the boronic acid group was protected by 1,3-propanediol, and then the hydroxy group was reacted with *N*,*N*'-disuccinimidyl carbonate (DSC). In the purification process using silica-gel chromatography, we collected compound **2** without 1,3-proponediol. The detailed procedure of synthesis, ^{1}H-NMR, and mass spectra are presented in the Supporting Information (Figures S1–S3).

BPmoc-Ins was prepared through a reaction between compound **2** and the amino residues of Ins by referring to our previous report.[29] BPmoc-Ins was purified *via* dialysis against water, and then it was lyophilized and stored in −20°C.

Scheme 2. Synthesis of an active ester of BPmoc: compound **2**.



Mass spectrometry

To characterize BPmoc-Ins, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used. The molecular weight of Ins is 5808 Da, and its mass spectrum displayed a peak of *m/z* at 5809, which agrees with $[M + H]^+$ (Figure S4). Because Ins has three primary amino groups, three peaks in the mass spectrum representing BPmoc-Ins might be seen; however, only one peak was observed. The molecular weights of mono-, di-, and tri-BPmoc–modified Ins are 5968, 6164, and 6342 Da, respectively. However, the mass spectrum of the prepared BPmoc-Ins had only one major peak at 6238 (Figure S5), in disagreement with the three aforementioned molecular weights. The *m/z* value of 6238 was larger than the molecular weight of di-BPmoc–modified Ins (6164 Da); therefore, we expected that the prepared BPmoc-Ins contains mainly tri-BPmoc–modified Ins.

We postulated that the observed *m/z* value of 6238 was derived from a decomposition peak of tri-BPmoc–modified Ins because some previous reports found decomposition peaks of phenylboronic acid-modified compounds in the mass spectra.[30,31] Crumpton *et al.* reported that some diol compounds effectively prevented undesired degradation of phenylboronic acid-modified peptides in

MALDI-TOF MS.[31] According to their report, we selected pinacol as an additive for samples for MALDI-TOF MS. The mass spectrum of the sample containing BPmoc-Ins and pinacol featured four main peaks (Figure S6). The largest *m/z* value was 6588. When three pinacol molecules bind to tri-BPmoc–modified Ins, the *m/z* value is expected to be 6589 ([tri-BPmoc–modified Ins + 3pinacol − $6H_2O$ + H]$^+$), which substantially corresponds to the measured *m/z* value of 6588.

In the mass spectrum (Figure S6), there were three other *m/z* peaks at 6471, 6355, and 6240. We assumed that these three peaks were derived from tri-BPmoc–modified Ins containing BPmoc groups uncovered with pinacol because phenylboronic acid induces undesired degradation.[30,31] The peaks at 6471, 6355, and 6240 were derived from a degraded compound from [tri-BPmoc–modified Ins + 2pinacol − $4H_2O$] (6506 Da), [tri-BPmoc–modified Ins + pinacol − 2 H_2O] (6423 Da), and tri-BPmoc–modified Ins (6342 Da), respectively. Thus, all four main peaks in Figure S6 can be assigned to tri-BPmoc–modified Ins, which supports our estimation that the main component of the prepared BPmoc-Ins is tri-BPmoc–modified Ins.

Next, we investigated whether Ins is generated from BPmoc-Ins by H_2O_2. In the mass spectrum of the sample of BPmoc-Ins with 10 mM H_2O_2, the main peak was found at 5808 (Figure S7), which substantially corresponds to the expected value of [Ins + H]$^+$ (5809). In the mass spectrum, another peak was found at 5958 that is probably derived from an intermediate in the conversion of BPmoc-Ins to Ins. One possible intermediate is an Ins analog containing one appended phenol residue (5958 Da, Figure S8).

Animal study

For animal experiments, we attempted to prepare a solution for injection; however, BPmoc-Ins was difficult to dissolve or disperse in pure water even after sonication. Some solid particles were found at the bottom of the vial (Figure 1a). We tried to dissolve BPmoc-Ins by adjusting the pH with HCl or NaOH, but this was ineffective. Then, we tested some additives for injection solutions and found that γ-cyclodextrin (CyD) effectively enhanced the dispersion of BPmoc-Ins (Figure 1b). The irradiation by a 532nm laser demonstrated the existence of the particles in the solution (b). Dimethylformamide was used as a good solvent for BPmoc-Ins (Figure 1c). For the dispersing ability, γ-CyD was selected as an additive for BPmoc-Ins injection.

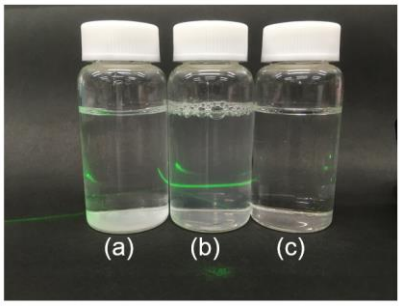


Figure 1. Image of BPmoc-Ins (1.0 mg) in solutions (10 mL) irradiated by a 532 nm laser; (a) in water; (b) in 161 mg/mL γ-CyD solution; (c) in dimethylformamide.

The animal study was performed in accordance with the animal use guidelines approved by the Life Science Research Center, Josai University (No. JU 19108). The injection was subcutaneously administered at 1.0 mL/kg into the backs of Goto–Kakizaki (GK) rats (n = 3), a model animal for

type II diabetes.[32–35] Glc levels were monitored in the animals using a flash Glc monitoring system (FreeStyle Libre system, Abbott Japan Co., Ltd.).[36] Although the FreeStyle Libre system was developed for humans, some reports described its applicability for assessments in certain animals.[37–39] In this study, we applied the sensor on the shaved back of the head of each GK rat (Figure 2) to monitor Glc levels.

Figure 2. A GK rat with an applied FreeStyle Libre sensor.

First, we inspected whether BPmoc-Ins itself exerts blood Glc-lowering effects. The formulation containing BPmoc-Ins (0.10 mg/mL) and γ-CyD (161 mg/mL) was subcutaneously administered at 1.0 mL/kg into the backs of GK rats. Figure 3a presents the Glc profile of GK rats after the injection of BPmoc-Ins without GOx. The increase of Glc levels in the first hour after administration was attributable to the effect of inhaled isoflurane, which was used immediately before the subcutaneous injection. During the first hour, Glc levels gradually decreased and reached a stable level of approximately 200 mg/dL, in line with the expected levels in GK rats.[32–35] A Glc level of 200 mg/dL is indicative of diabetes, suggesting that the blood Glc-lowering effect of Ins was sealed by BPmoc. Human Ins has three amino residues that can be modified: A-chain Gly1, B-chain Phe1, and B-chain Lys29. Ins analogs modified at B-chain Lys29 demonstrate sufficient Glc-lowering activity.[40,41] In contrast, the modification at Gly1 of the A-chain has a significant impact on the reduction of insulin

activity.[42,43] Because BPmoc-Ins is modified at all three amino residues, the Ins activity of BPmoc-Ins is completely sealed (Figure 3a).

In the same manner, the formulation containing BPmoc-Ins (0.10 mg/mL), GOx (0.10 mg/mL), and γ-CyD (161 mg/mL) was subcutaneously administered to GK rats. Although we were concerned about irritation associated with H_2O_2-produced GOx, we confirmed the absence of inflammation at the site of injection. Figure 3b reveals that the formulation containing BPmoc-Ins and GOx sharply decreased blood Glc levels in the first 2 h after injection, in contrast to effects of the formulation lacking GOx. Figure 3c highlights the significant difference of the lowest Glc concentration of each condition between the presence or absence of GOx. The Glc profiles in Figure 3b strongly support the conversion of BPmoc-Ins into Ins in a living animal.

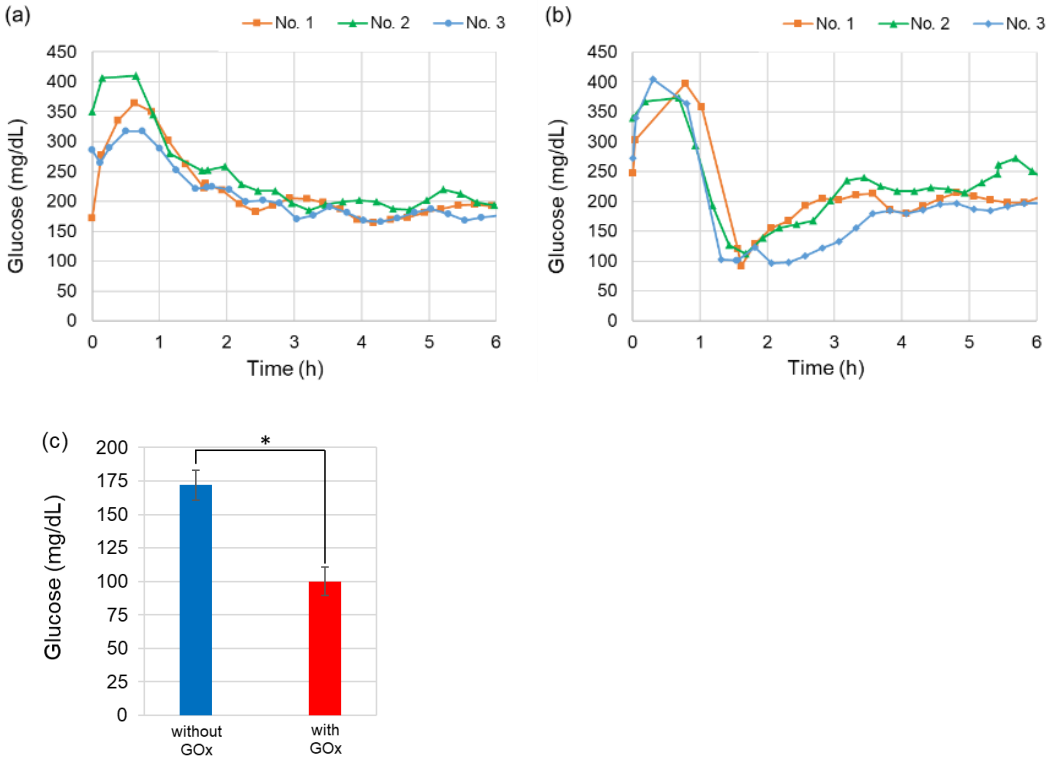


Figure 3. Profiles of blood Glc levels in GK rats (n = 3) after subcutaneous injection (1.0 mL/kg) of formulations containing BPmoc-Ins (0.10 mg/mL) and γ-CyD (161 mg/mL): (a) without GOx; (b) with GOx (0.10 mg/mL); (c) The averages of the lowest Glc levels (n = 3) during 1–6 h after administration for each condition. * $p < 0.01$ as calculated using the paired Student's t-test.

Two more formulations were investigated as control experiments. The first formulation containing Ins (0.10 mg/mL) and γ-CyD (161 mg/mL) lowered the Glc level in GK rats (Figure S9a). This result demonstrates that the evaluation method with the FreeStyle Libre system is appropriate to evaluate the Glc-lowering effect of Ins. The other formulation containing GOx (0.10 mg/mL) and γ-CyD (161 mg/mL) did not demonstrate the Glc-lowering effect (Figure S9b). Although some reports show inflammation caused by GOx,[44,45] and there were no signs of skin inflammation after the injection of GOx and γ-CyD (Figure S10).

The control experiments using the two formulations support that the Glc-lowering effect observed in Figure 3b was due to Ins converted from BPmoc-Ins.

High-performance liquid chromatography (HPLC) study

To add further evidence of the conversion of BPmoc-Ins into Ins, we conducted an HPLC analysis. We prepared buffered samples (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid: HEPES, pH 7.4) containing BPmoc-Ins (0.10 mg/mL), GOx (0.10 mg/mL), γ-CyD (161 mg/mL), and various

concentrations of Glc. In the chromatogram of a sample without Glc (Figure 4a), there was a main peak at 13 min that was derived from BPmoc-Ins. Samples containing Glc were incubated for 1.0 h at 37°C before injection into the HPLC system. In the chromatogram of a sample containing 5.0 mM Glc (Figure 4b), the peak at 13 min was smaller, and several new peaks appeared between 9 and 13 min. These peaks were probably derived from decomposition intermediates such as di-BPmoc–modified Ins, mono-BPmoc–modified Ins, or phenol-appended Ins, as presented in Figure S8. In the chromatogram of a sample containing 20 mM Glc (Figure 4c), the large peak observed at around 9 min corresponded to unmodified Ins, demonstrating that the conversion of BPmoc-Ins into Ins successfully occurs under this condition.

It is noteworthy to compare the chromatograms in Figure 4 because they prove that the generation of Ins from BPmoc-Ins is dependent on the Glc concentration. The Glc concentration used in Figure 4c was 20 mM (360 mg/dL), a patient with 20 mM Glc would be diagnosed as having diabetes.[20] The chromatogram of Figure 4c clearly illustrates the conversion of BPmoc-Ins into Ins. In addition, the animal test data in Figure 3b strongly support that the generation of Ins from BPmoc-Ins occurs in the presence of high Glc levels. On the contrary, the Glc concentration of 5.0 mM (90 mg/dL) used in Figure 4b is similar to the Glc level of healthy people between meals.[20] At a relatively low Glc level such as that observed in patients with diabetes in a state of hunger, an injection of unmodified Ins induces hypoglycemia. However, we assume that the injection containing BPmoc-Ins and GOx has the potential to prevent abnormally low Glc levels because the conversion

of BPmoc-Ins into Ins is slow at low Glc levels, as supported by the result of Figure 4b. To prove this assumption, we need appropriate animal conditions, which will be our next challenge.

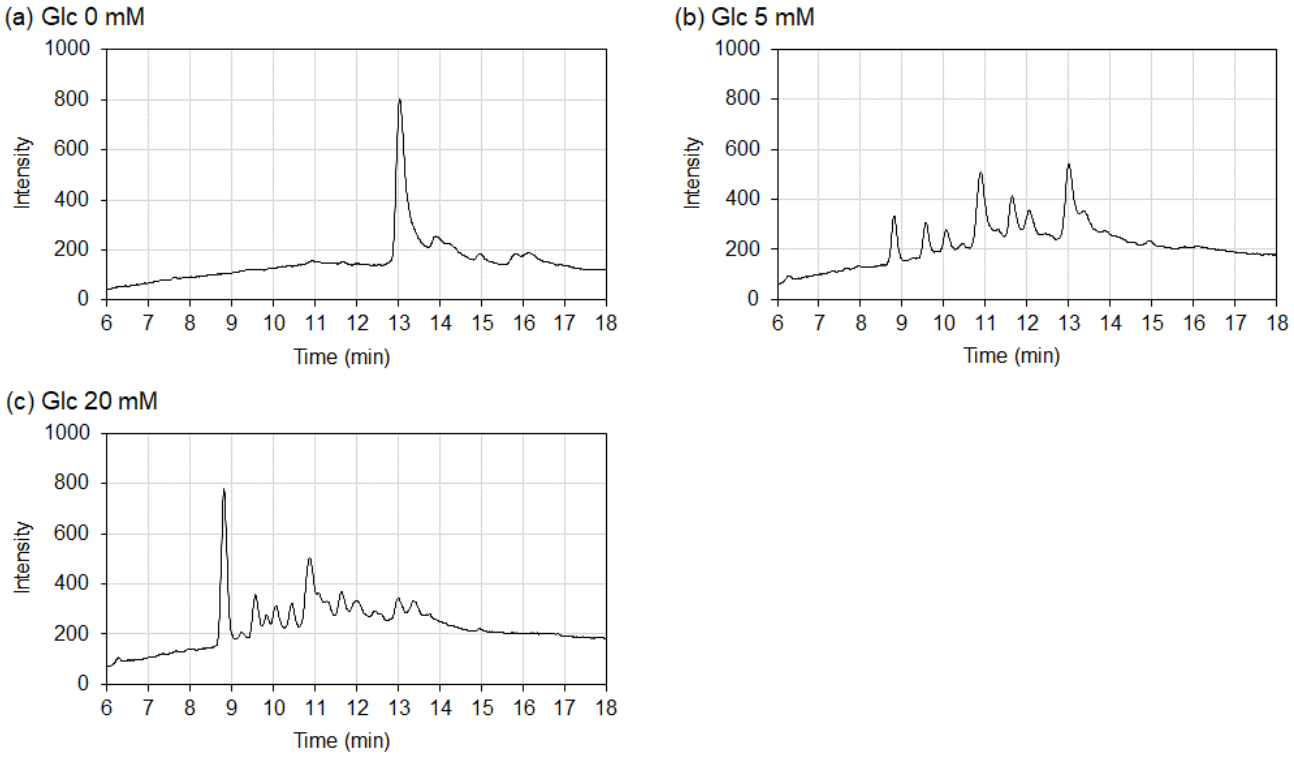


Figure 4. HPLC chromatograms of samples containing BPmoc-Ins (0.10 mg/mL), GOx (0.10 mg/mL), and γ-CyD (161 mg/mL): (a) without Glc; (b) incubated with 5.0 mM Glc for 1.0 h at 37°C; and (c) incubated with 20 mM Glc for 1.0 h at 37°C.

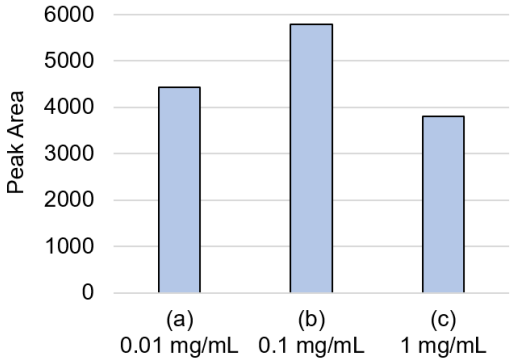


Figure 5. The peak areas of Ins in the chromatogram of samples containing GOx (various

concentrations), BPmoc-Ins (0.10 mg/mL), γ-CyD (161 mg/mL), and Glc (20 mM) after incubation for 1 h at 37°C.

The balance between GOx and BPmoc-Ins is important because the amount of GOx affects the conversion ratio of BPmoc-Ins into Ins. We conducted an additional HPLC study with varying concentration of GOx (0.010, 0.10, and 1.0 mg/mL). Figure 5 summarizes the peak areas of Ins at each condition. The peak area did not increase with increasing GOx concentration. This unexpected result may be attributed to a pH decrease in gluconic acid production accompanied by Glc oxidation and the decrease in pH that lowered the activity between BPmoc-Ins and H_2O_2.

This postulation was verified by the following experiments. We prepared a sample containing Glc (20 mM) and GOx (1.0 mg/mL) with HEPES buffer (10 mM, pH 7.4) and measured its pH value through 1 h of incubation at 37ºC. Our result showed that the pH value decreased from 7.4 to 5.9. Next, we prepared two samples containing H_2O_2 (10 mM), BPmoc-Ins (0.10 mg/mL), and HEPES (10 mM), and adjusted their pH to 6.0 and 7.4, respectively. After 1 h of incubation at 37ºC, the samples were analyzed by HPLC. The chromatograms show that the Ins peak (around 9 min) of the pH 6.0 sample was much smaller than that of the pH 7.4 sample (Figure S11).

These results suggest that excessive GOx induces pH decrease, which causes lowering of the activity between BPmoc-Ins and H_2O_2. Some previous reports have effectively utilized the pH decrease by GOx for Glc-triggered drug release systems;[46,47] however, the lower pH interfered with

our system. Among the conditions shown in Figure 5, the sample containing 0.10 mg/mL GOx produced the largest amount of Ins; this finding supports the validity of animal tests using 0.10 mg/mL GOx (Figure 3b).

Conclusion

In summary, we prepared BPmoc-Ins and confirmed that the directly attached BPmoc groups effectively sealed the blood Glc-lowering effect of Ins (Figure 3a). A unique point of our approach is the co-administration of GOx with BPmoc-Ins as an injection. The co-injected GOx effectively promoted the conversion of BPmoc-Ins into Ins, and then the generated Ins reduced blood Glc levels, which was confirmed in animal tests (Figure 3b, c). Our strategy only requires mixing two components; *i.e.,* we did not use any well-designed drug carrier or support for Glc-responsive Ins release systems. This approach permits easy preparation of the formulation and adjustment of the balance of its two components: a BPmoc-modified drug and a transducer such as GOx. The ease of this approach will expand the potential development of stimuli-responsive drug release systems.

Supporting Information

*Supporting Information: The Supporting Information is available free of charge on the ACS Publications website at DOI:

Detailed information about materials and experimental methods including synthesis, ^{1}H NMR

spectra, MS spectra, MALDI-TOF MS, HPLC, and animal studies.

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Conflict of Interest Disclosure

The authors declare no competing financial interest.

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Supporting Information

Hydrogen peroxide-triggered conversion of boronic acid-appended insulin into insulin and its application as a glucose-responsive insulin formulation

*Hinako Kikuchi, Yuki Nakamura, Chika Inoue, Sayaka Nojiri, Miho Koita, Minori Kojima, Hiroki Koyama, Ryotaro Miki, Toshinobu Seki, Yuya Egawa**

Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan

Contents

EXPERIMENTAL

Materials

Acetonitrile (AcCN) for high-performance liquid chromatography (HPLC), AcCN for liquid chromatography-mass spectrometry (LC-MS), AcCN (super-dehydrated), *N,N*-dimethylformamide (DMF, super-dehydrated), dimethyl sulfoxide (DMSO, super-dehydrated), *N,N*'-disuccinimidyl carbonate (DSC), D-glucose (Glc), hydrogen peroxide (H_2O_2, 30% aqueous solution), insulin (Ins, human, recombinant, potency > 27.5 U/mg), tetrahydrofuran (THF, super-dehydrated, stabilizer-free), triethylamine, and trifluoroacetic acid (TFA) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Ammonium acetate, α-cyano-4-hydroxycinnamic acid (CHCA), DMSO-d_6, glucose oxidase (from *Aspergillus niger* Type X-S, lyophilized powder, 100,000–250,000 units/g solid without added oxygen), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and poly(ethylene glycol) (PEG, analytical standard, for GPC, 6000) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Tributylamine, pinacol, and 1,3-propanediol were bought from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). γ-Cyclodextrin (γ-CyD) was purchased from Junsei Chemical Co. Ltd. (Tokyo, Japan). 4-(Hydroxymethyl)phenylboronic acid was bought from Combi-Blocks Inc. (San Diego, CA, USA). Isoflurane (Japanese Pharmacopoeia) was purchased from Pfizer Japan Inc (Tokyo, Japan). All other chemicals were of at least reagent grade and were used as received.

Synthesis

Synthesis of compound 1. Compound **1** was synthesized according to a previously reported method.[1] 4-(Hydroxymethyl)phenylboronic acid (2.0 g, 13 mmol) and 1,3-propanediol (1.0 g, 13 mmol) were dissolved in THF (super-dehydrated, 40 mL), and the solution was stirred for 3 days at room temperature. The solvent was evaporated under reduced pressure, and the viscous colorless liquid was purified via silica gel column chromatography with hexane/ethyl acetate (6:4, v/v) to afford compound **1** as white powder (0.66 g, 3.7 mmol, 28%). ^{1}H-NMR (400 MHz with Varian

400-MR, DMSO-d_6, Figure S1) δ 7.60 (d, 2H), 7.26 (d, 2H), 5.18 (t, 1H), 4.48 (d, 2H), 4.08 (t, 4H), 1.98 (quint., 2H). There were some small peaks derived from 4-(hydroxymethyl)phenylboronic acid and 1,3-propanediol because a certain amount of compound **1** was dissociated in DMSO-d_6.

Synthesis of compound 2. Compound **2** was synthesized by referring to a previously reported method.[2] Compound **1** (0.66 g, 3.4 mmol) was dispersed into super-dehydrated AcCN (40 mL). Then, DSC (1.3 g, 5.0 mmol) and triethylamine (1.4 mL) were added to the solution, and the reaction solution was stirred under nitrogen atmosphere at room temperature for 2.5 h. After the reaction solution was transferred to a separatory funnel, chloroform (50 mL) was added. The organic solution was washed with distilled water (30 mL × 3). The organic layer was separated, dried with anhydrous $MgSO_4$, and filtered. The filtrate was evaporated, and the residue was subjected to silica gel column chromatography with chloroform/ethyl acetate (20:1, v/v) to obtain compound **2**, which lacks 1,3-propanediol, as white powder (0.20 g, 0.67 mmol, 18%). ^{1}H-NMR (400 MHz, DMSO-d_6, Figure S2), δ 8.12 (s, 2H), 7.81 (d, 2H), 7.39 (d, 2H), 5.39 (s, 2H), 2.80 (s, 4H). MS (FAB, negative mode, matrix: glycerol, Figure S3) *m/z*: 348 ([M + glycerol − $2H_2O$ − H]$^-$ requires 348). In the MS spectrum, compound **2** was detected as a cyclic ester composed of compound **2** and glycerol.

Preparation of *p*-boronophenylmethoxycarbonyl (BPmoc)-Ins. Modification of Ins was performed in line with our previous report.[3] Ins (0.30 g, 52 μmol) was dissolved in a mixture of tributylamine (300 μL) and DMSO (60 mL). Separately, compound **2** (86 mg, 0.29 mmol) was dissolved in a mixture of tributylamine (61 μL) and DMF (12 mL). The compound **2** solution was added dropwise into the Ins solution, and the reaction solution was stirred at room temperature under a nitrogen atmosphere for 3 h. Then, water (10 mL) was added to stop the reaction, and the solution was dialyzed against water (1 L, nine times) using a dialysis tube (molecular weight cut-off, 3500). The resulting solution was lyophilized, and BPmoc-Ins was obtained (0.22 g).

Mass Spectrometry

AcCN for LC-MS and an aqueous solution containing 0.10% TFA were mixed in a 1:1 ratio (v/v), and this solution was used to prepare a matrix solution containing 5.0 mg/mL CHCA and a sample solution containing 0.10 mg/mL BPmoc-Ins. The matrix and sample solutions were mixed in a 1:1 ratio (v/v), and the mixture was spotted on the plate for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. Ins was also analyzed using the same procedure. PEG was used for a mass standard to calibrate the mass spectrometer (JMS-S3000, JEOL Ltd., Tokyo, Japan).

Confirmation of the modification of BPmoc groups. To protect BPmoc groups with pinacol, an alkaline condition was used. An aqueous solution containing 10 mM ammonium acetate was adjusted to pH 9.5 by adding a small amount of aqueous NaOH solution. This solution was used to prepare 0.10 M pinacol solution. The pinacol solution, the aforementioned sample solution containing 0.10 mg/mL BPmoc-Ins, and the matrix solution containing 5.0 mg/mL CHCA were mixed at a 1:1:1 ratio (v/v/v). The mixture was spotted to the plate for MALDI-TOF MS.

Confirmation of reactivity with H_2O_2. To facilitate the reaction between BPmoc and H_2O_2, an alkaline condition was used. A small amount of aqueous NaOH solution was added to the aforementioned sample solution containing 0.10 mg/mL BPmoc-Ins adjusted to pH 9.5. Next, a small amount of 30% H_2O_2 aqueous solution was added to the sample solution to a final concentration of 10 mM. This sample solution was mixed with the matrix solution containing 5.0 mg/mL CHCA in 1:1 (v/v), and the mixture was spotted to the plate for MALDI-TOF MS.

Animal studies

Animal studies were performed in accordance with the animal use guidelines approved by the Life Science Research Center, Josai University (No. JU 19108 and JU 21087). Male Goto–Kakizaki (GK) rats were obtained from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). To monitor

blood Glc levels, a continuous Glc monitoring system (FreeStyle Libre system, Abbott Japan Co., Ltd., Tokyo Japan) was used. GK rats were anesthetized with inhaled isoflurane, and the back of the head of each animal was shaved to attach the sensor device (5 g, 35 mm diameter × 5 mm thickness). The sensor device was fixed and held by surgical tapes, and a small amount of instant glue was used to bond the surgical tape and sensor device (**Figure 2**). The FreeStyle Libre reader was placed closer to the sensor device to capture the continuous Glc concentration data from the sensor.

To prepare the injection formulation, ultra-pure water was used. An aqueous solution containing γ-CyD (161 mg/mL) was prepared. BPmoc-Ins (0.10 mg/mL) was added to the γ-CyD solution, and the mixture was sonicated for 5 min to disperse BPmoc-Ins. This injection formulation was subcutaneously administered at 1.0 mL/kg into the backs of GK rats immediately after anesthetization with inhaled isoflurane. The Glc concentration was monitored using FreeStyle Libre for 6 h in the fasted condition. To prepare the formulation containing GOx, BPmoc-Ins (0.10 mg/mL) and GOx (0.10 mg/mL) were added to the γ-CyD solution (161 mg/mL), and the mixture was sonicated for 5 min to disperse BPmoc-Ins. The injection formulation containing GOx was administered to GK rats under the same condition. The statistical significance of differences in the results was analyzed using a paired Student's *t*-test ($p < 0.01$) for comparisons between two groups.

HPLC study

HPLC analysis was conducted using an octadecylsilyl column (5 μm, 150 × 4.6 mm i.d., CAPCELL PAK C18, Shiseido Fine Chemicals, Tokyo, Japan) and gradient elution at a flow rate of 1.0 mL/min. Mobile phase A consisted of AcCN/water/TFA (300:700:1, v/v/v), and mobile phase B was composed of AcCN/water/TFA (950:50:1, v/v/v). The HPLC system (LC-20AT, SPD-20A, Shimadzu Corporation, Kyoto, Japan) was operated in the gradient mode with a linear increase from 0 to 33.3% for mobile phase B over 20 min. Elution was monitored by the absorption at 280 nm.

To prepare samples for HPLC analysis, we used buffer solution (pH 7.4) containing 10 mM

HEPES. BPmoc-Ins (0.10 mg/mL), GOx (0.10 mg/mL), and γ-CyD (161 mg/mL) were added to the buffer solution, and the mixture was sonicated for 5 min. Then, Glc was added at varying concentrations. In the case of 5.0 and 20 mM Glc, the samples were incubated for 1.0 h at 37°C. Before injection into the HPLC system, the samples were filtered through a hydrophilic polytetrafluoroethylene membrane with a pore size of 0.20 μm (DISMIC®-13HP, Advantec Toyo Kaisha, Ltd. Japan).

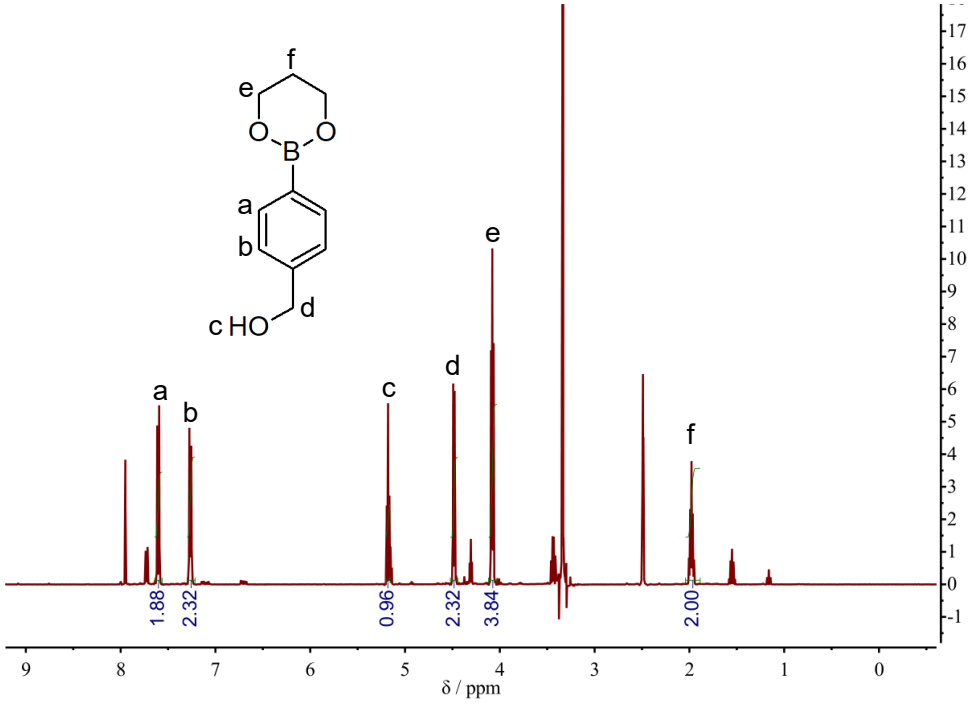


Figure S1. The ^{1}H-NMR spectrum of compound **1** in DMSO-d_6.

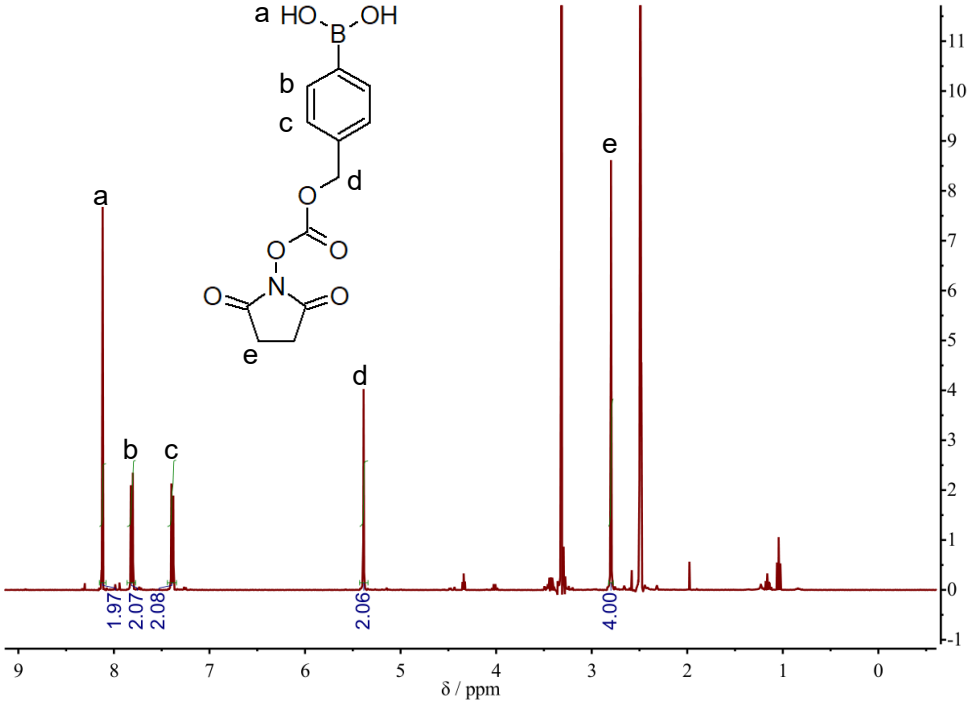


Figure S2. The ^{1}H-NMR spectrum of compound **2** in DMSO-d_6.

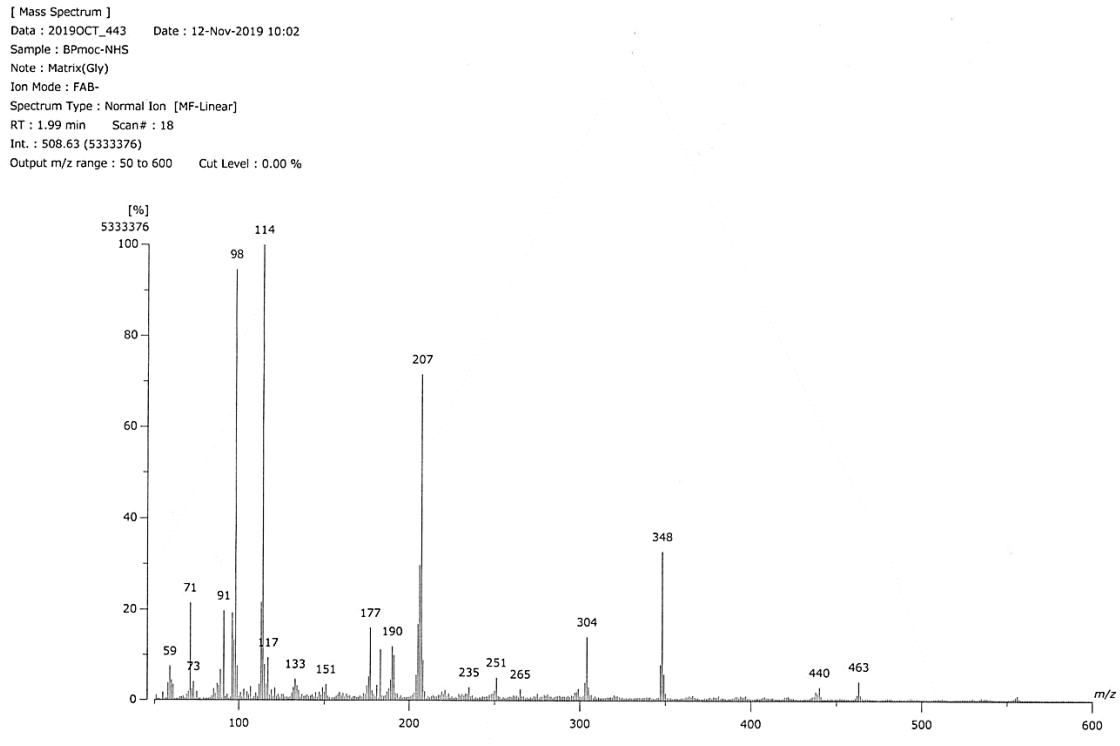


Figure S3. Mass spectrum of compound **2** (FAB, negative mode, matrix: glycerol). A peak was observed at 348. The expected *m/z* value of $[M + \text{glycerol} - 2H_2O - H]^-$ is 348.

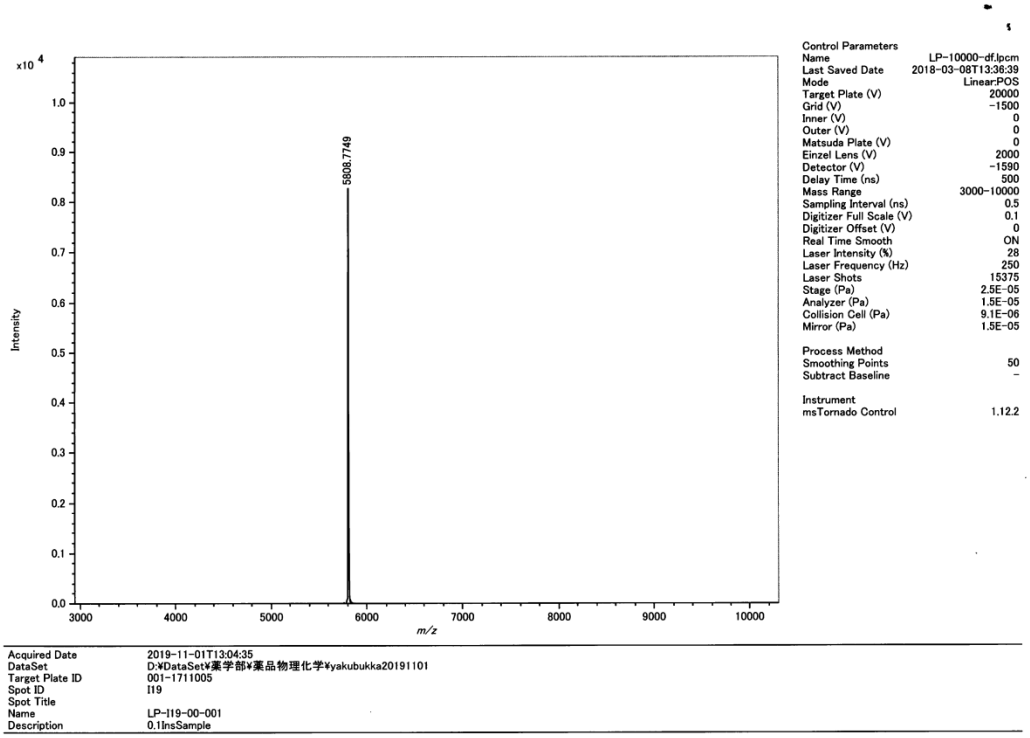


Figure S4. Mass spectrum of Ins (MALDI-TOF, positive mode, matrix: CHCA). A peak was observed at 5809. The expected *m/z* value of $[M + H]^+$ is 5809.

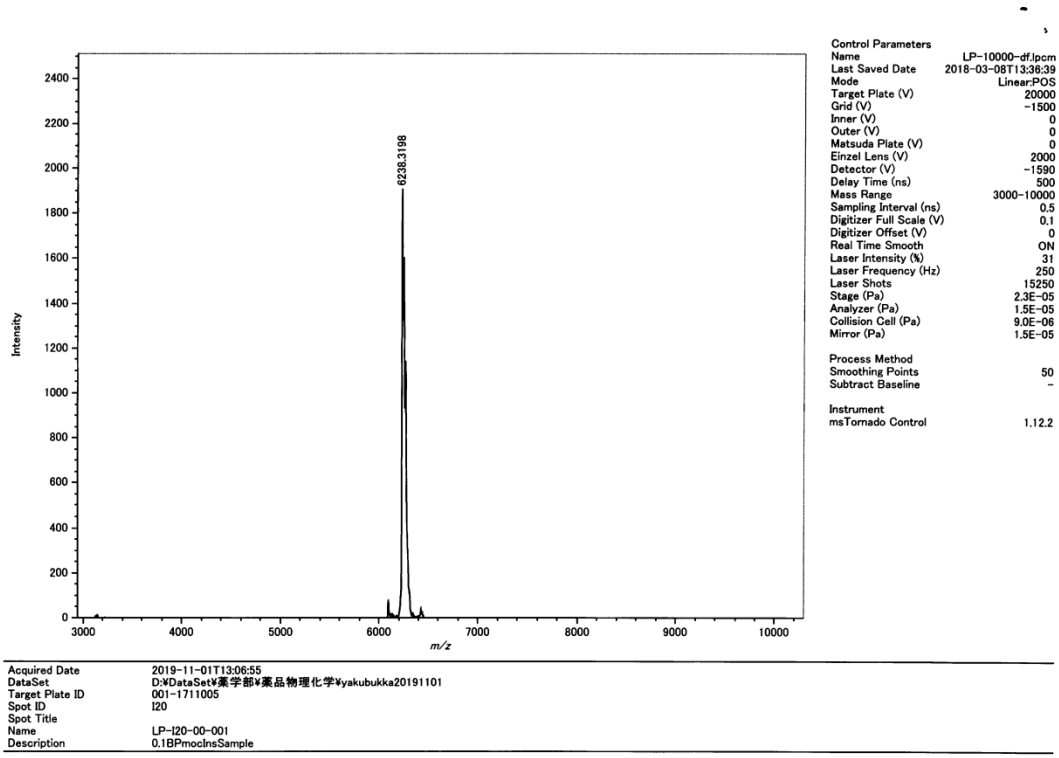


Figure S5. Mass spectrum of BPmoc-Ins (MALDI-TOF, positive mode, matrix: CHCA). A peak was observed at 6238. The expected *m/z* value of $[\text{BPmoc-Ins} + \text{H}]^+$ is 6343 when three BPmoc groups are present.

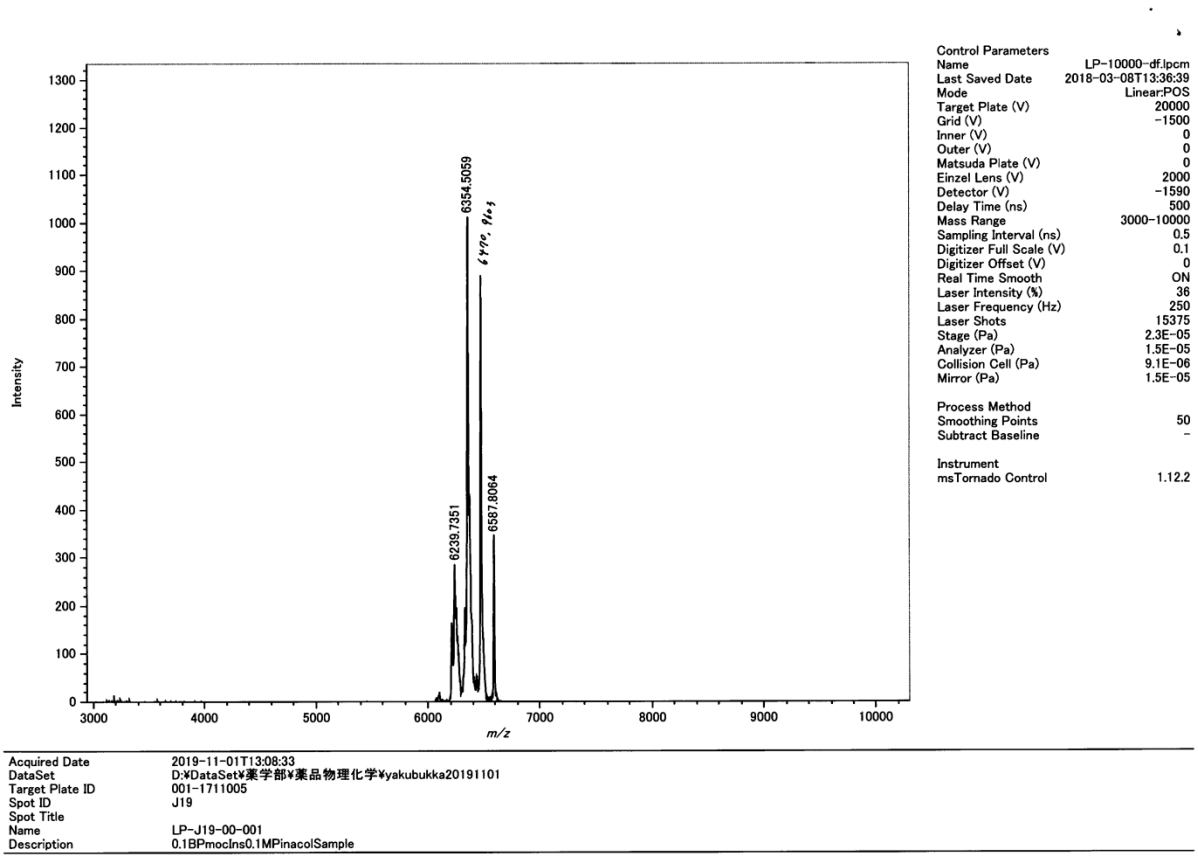


Figure S6. Mass spectrum of BPmoc-Ins with pinacol (MALDI-TOF, positive mode, matrix: CHCA). Four peaks were observed at 6588, 6471, 6355, and 6240. The expected *m/z* value of [BPmoc-Ins + 3pinacol – $6H_2O$ + H]$^+$ is 6589 when Ins is modified by three BPmoc groups.

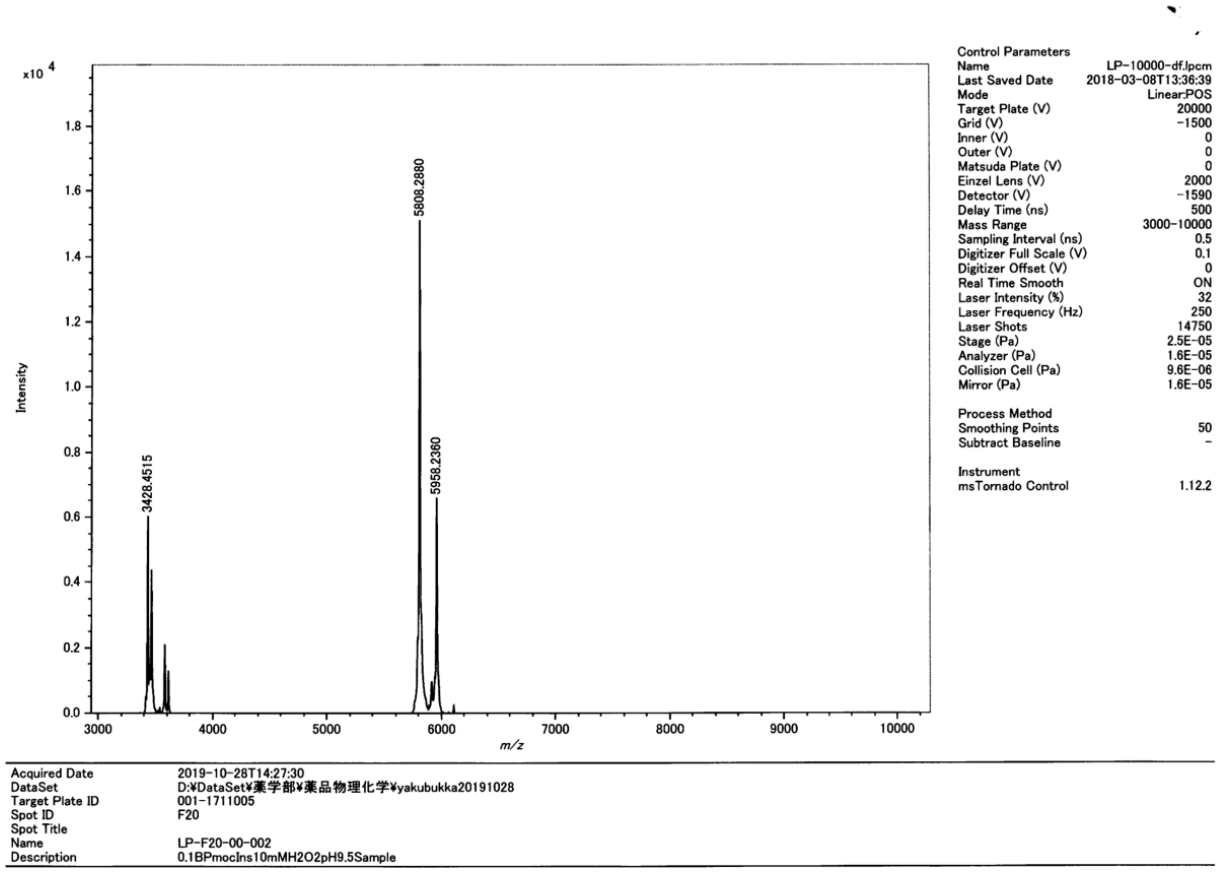


Figure S7. Mass spectrum of the solution of BPmoc-Ins with H_2O_2 at pH 9.5 (MALDI-TOF, positive mode, matrix: CHCA). A peak was observed at 5808. The expected *m/z* value of $[Ins + H]^+$ is 5809.

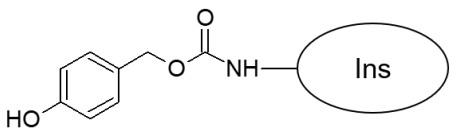


Figure S8. Structure of an insulin analog containing one appended phenol residue (5958 Da).

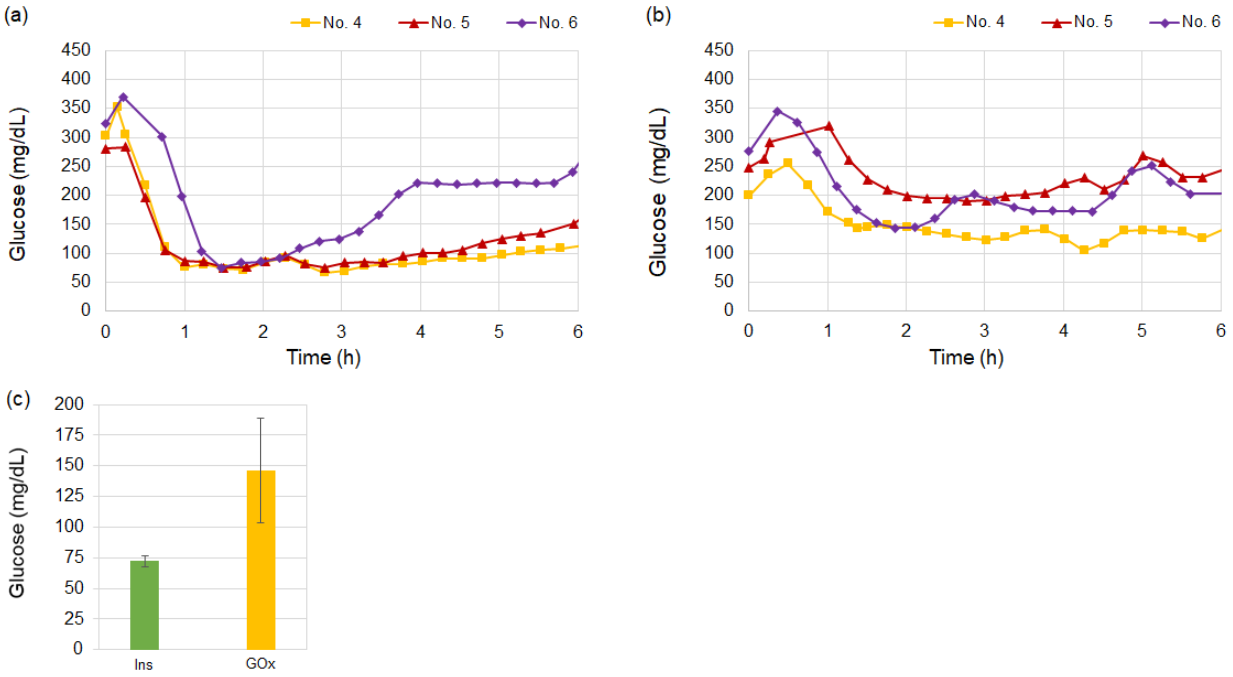
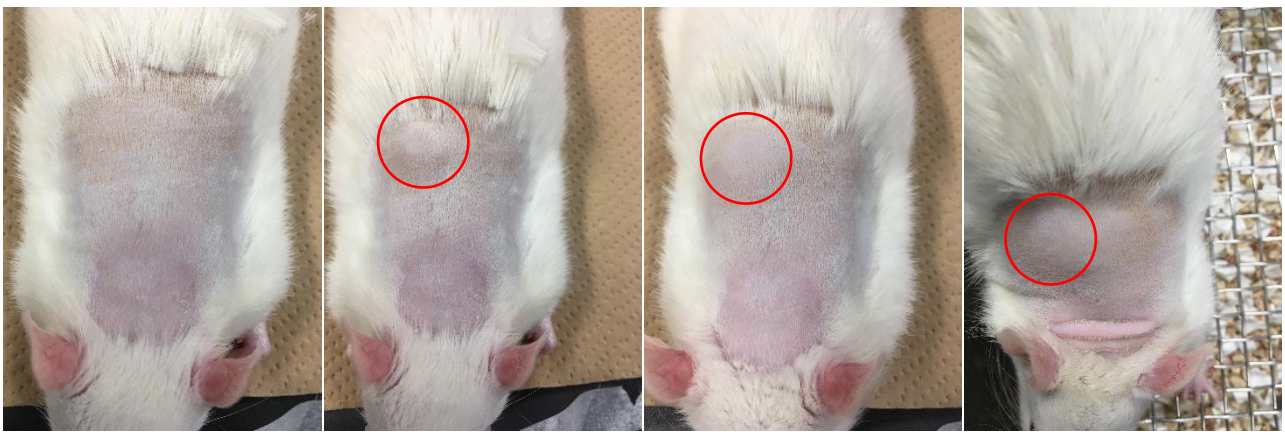


Figure S9. Profiles of blood Glc levels in GK rats (n = 3) after the subcutaneous injection (1.0 mL/kg) of formulations containing γ-CyD (161 mg/mL): (a) with Ins (0.10 mg/mL); (b) with GOx (0.10 mg/mL); (c) The lowest average Glc levels (n = 3) during 1–6 h from the administration for each condition.



(a) (b) (c) (d)

Figure S10. Pictures of a GK rat before and after the administration of GOx. The red circle shows the injected part: (a) before injection, (b) immediately after the injection, (c) after 1 h from the injection, and (d) after 6 h from the injection.

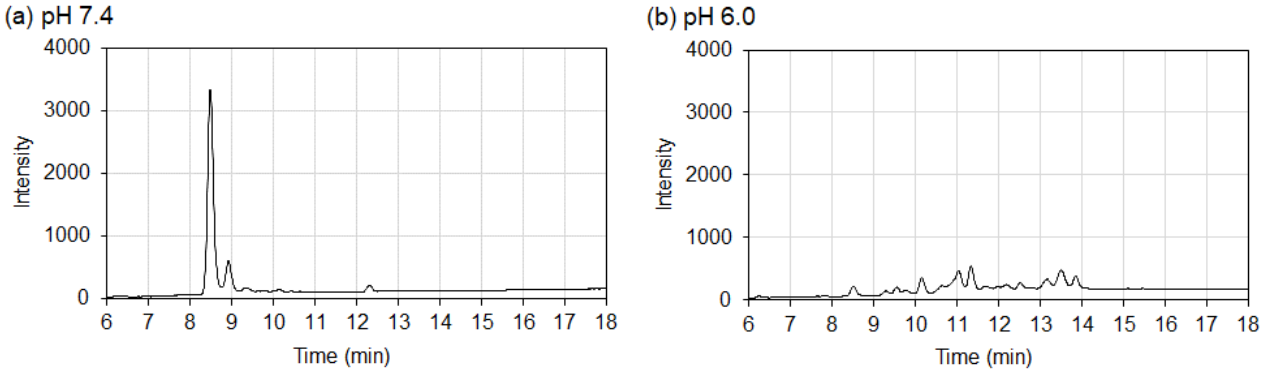


Figure S11. HPLC chromatograms of samples containing H_2O_2 (10 mM), BPmoc-Ins (0.10 mg/mL), γ-CyD (161 mg/mL), and HEPES (10 mM) incubated for 1 h at 37°C: (a) in pH 7.4 solution and (b) in pH 6.0 solution.

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