ACS OMEGA

Article

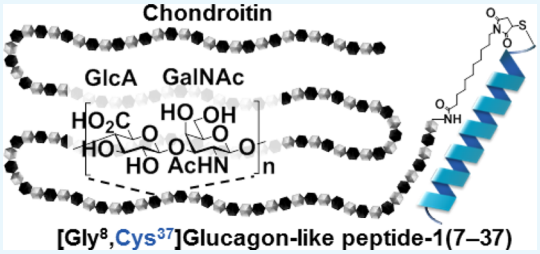
Cite This: ACS Omega 2018, 3, 5346−5354

Glycosaminoglycan Conjugation for Improving the Duration of Therapeutic Action of Glucagon-Like Peptide-1

Megumi Ichikawa, Tetsuya Hirayama, Masanobu Fukushima, Ikue Kitazawa, Kazuhiro Kojima, Tokiko Sakai, Yoshihiro Takatsu,* and Tetsuya Ohtaki

Central Research Laboratories, Seikagaku Corporation, Tateno 3-1253, Higashiyamato-shi, Tokyo 207-0021, Japan

ABSTRACT: Glucagon-like peptide-1 (GLP-1) is an incretin peptide that plays a crucial role in lowering blood glucose levels and holds promise for treating type II diabetes. In this study, we synthesized GLP-1 derivatives that were conjugated with glycosaminoglycans (GAGs), i.e., chondroitin (CH) or heparosan (HPN), to address the major limitation in their clinical use, which is their short half-life in the body. After exploring a variety of CHs with different molecular sizes and heterobifunctional linkers having different alkyl chains, we obtained CH-conjugated GLP-1 derivatives that stayed in blood circulation much longer ($T_{1/2\ elim}$ > 25 h) than unconjugated GLP-1 and showed blood glucose-lowering efficacy up to 120 h after subcutaneous injection in mice. By using the same optimized linker design, we eventually obtained a HPN-conjugated GLP-1 derivative with efficacy lasting 144 h. These results demonstrate that conjugation with GAG is a promising strategy for improving the duration of peptide drugs.



INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is an incretin peptide that is secreted from intestinal L-cells in response to orally ingested nutrients. It lowers plasma glucose levels by potentiating glucose-dependent insulin secretion from the endocrine pancreas with very low risk of hypoglycemia. In addition to its blood glucose-lowering activity, GLP-1 exerts inhibitory effects on gastric emptying, gastric acid release, glucagon secretion, and food intake.[1−5] Because of these physiological actions, GLP-1 is a promising therapeutic target for treating noninsulin-dependent diabetes mellitus (NIDDM) and obesity.

However, GLP-1 is quite unstable in vivo; its half-life ($T_{1/2\ elim}$) is only 2 min or less when it is injected intravenously.[6] Accordingly, clinical application of GLP-1 requires frequent administration at high doses. To overcome this shortcoming, a large number of studies have attempted to address the causes of its degradation, such as inactivation by dipeptidyl peptidase IV (DPP IV) or other proteases, rapid disappearance by renal excretion, and hepatic metabolism.[7,8] The former issue is partially solved by replacement of the DPP IV-vulnerable residue (Ala[8]), and the latter is ameliorated by enlarging and shielding the peptide with inert materials. As of now, scientists and clinicians have successfully developed NIDDM therapeutics including an acylated GLP-1 derivative, liraglutide; a lizard-derived GLP-1 analogue, exenatide; and an Fc-fusion GLP-1, dulaglutide.[9−11]

Modification with polyethylene glycol (PEGylation) has also been shown to be beneficial for improving the pharmacokinetics (PK) of GLP-1 by forming a hydrated cloud that effectively protects GLP-1 from renal clearance and proteolysis.[12−15] However, PEGylation has some issues. First, PEG is not biodegradable, and repeated administration of PEGylated proteins can induce intracellular vacuolation.[16−18] Second, PEGylation of foreign immunogenic moieties such as heterogenic proteins often causes the production of anti-PEG antiserum, indicating that PEG is antigenic.[19−21] Even in naïve humans who have experienced no administration of PEGylated drugs, the prevalence of anti-PEG antibodies has been reported to be up to 25%.[22,23]

As an alternative to PEG, biodegradable polysaccharides such as hydroxyethyl starch and polysialic acid have been conjugated to protein drugs to extend their blood half-lives.[24−28] In addition, we have recently shown that chondroitin (CH), a type of glycosaminoglycan (GAG) composed of disaccharide repeats of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc), improves the PK of protein drugs.[29] CH-conjugated interferon fully retains its bioactivity, and its plasma activity lasts 100 h after intravenous (iv) injection.[29] CH-conjugated bacterial asparaginase preserves its enzymatic activity and does not raise anti-CH or antiasparaginase antibodies after repeated injection.[29] CH is less antigenic because of its abundance in the human body. Notably, large amounts of CH disaccharide repeats are found in human blood.[29]

In this study, CH or another GAG, heparosan (HPN), was conjugated via *N*-hydroxysuccinimide−maleimide linkers (i.e., heterobifunctional linkers) to the C-terminus of the GLP-1 peptide that had been modified with a cysteine residue. The conjugated peptides were evaluated in comparison with liraglutide for in vitro agonist activity, plasma circulation after iv and subcutaneous (sc) injections, and blood glucose-

Received: March 13, 2018
Accepted: May 8, 2018
Published: May 18, 2018

DOI: 10.1021/acsomega.8b00467
ACS Omega 2018, 3, 5346−5354

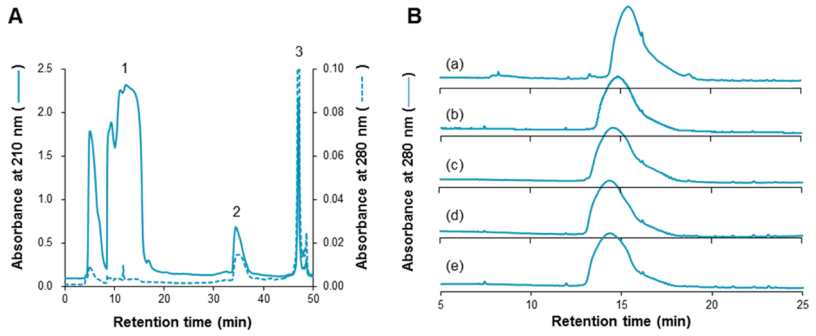


Figure 1. HPLC purification of CH-conjugated GLP-1C peptides. (A) Purification of CH140−EDA−(LMDS)−GLP-1C peptide using a preparative ODS HPLC column. Peak 1: mixture of CH140 and EDA-modified CH140; peak 2: CH140−EDA−(LMDS)−GLP-1C; and peak 3: GLP-1C. (B) Analytical HPLC of purified conjugated peptides CH70−EDA−(X)−GLP-1, where X = KMUS (a), HMCS (b), EMCS (c), GMBS (d), or BMPS (e). The peak top retention time was delayed with increasing carbon number of the tethering arm.

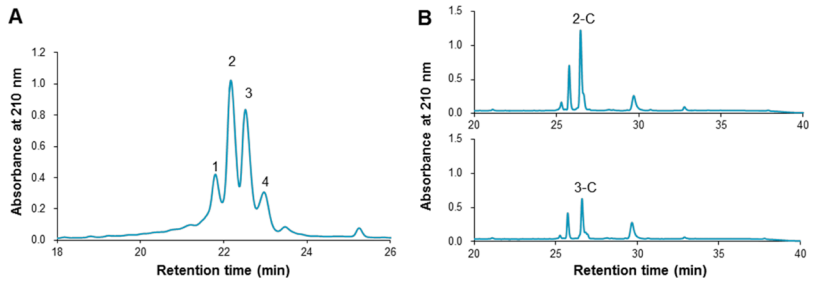


Figure 2. Digestion of CH-conjugated GLP-1C peptide. (A) HPLC purification of C-ABC-digested CH70−EDA−(HMCS)−GLP-1C. Peaks 2 and 3 were subjected to further digestion with Glu-C endopeptidase. Peaks 1 and 4 were directly subjected to MS/MS. (B) HPLC purification of Glu-C-digested peak 2 (upper) and peak 3 (lower). Peaks 2-C and 3-C were analyzed by MS/MS for structural identification.

lowering activity in mice. We demonstrate that the plasma half-lives of conjugated peptides are affected primarily by the chain length of the heterobifunctional linkers and that optimized conjugated peptides show robust blood glucose-lowering activity lasting 120−144 h postadministration.

RESULTS AND DISCUSSION

Design of Conjugated GLP-1 Peptide. The active form of GLP-1 is known as GLP-1(7−37) or GLP-1(7−36) amide, of which the N-terminal His residue is essential for receptor activation. Hence, we designed [Gly,[8] Cys[37]GLP-1(7−37) for conjugating the Cys residue of the peptide with amine-modified CH via heterobifunctional linkers in this study.

To ensure one-to-one and site-specific conjugation, CH was modified into amine-modified forms by reductive amination of the reducing end's sugar. The content of the amino groups ranged between 15 and 80% (mol/mol) depending on the diamines tested, i.e., ethylenediamine (EDA), hexanediamine (HDA), 1,4-bis(aminomethyl)cyclohexane (BAMC), and 1,4-bis(aminomethyl)benzene (BAMB). Among them, those with higher hydrophobicity were less efficient, and EDA was the best in terms of efficiency and robustness.

Preparation of CH-Conjugated GLP-1C Peptides. In our standard small-scale procedure, 40 mg of amine-modified GAG was modified with different types of heterobifunctional linkers and subsequently conjugated with [Gly,[8] Cys[37]GLP-1(7−37), designated as GLP-1C hereafter. The conjugate was purified by reversed-phase high-performance liquid chromatography (HPLC), as shown in Figure 1A. Separation of conjugated peptide from unconjugated CH and GLP-1C was quite successful; conjugated peptide was well-retained in the octadecylsilyl (ODS) column and was eluted earlier than GLP-1C, whereas CH was never retained in the column. In contrast, PEGylated GLP-1C was eluted later than GLP-1C, indicating that PEG is hydrophobic compared to CH.

Analytical HPLC was performed to examine the purity of conjugated peptides (Figure 1B). Intriguingly, the retention times of the conjugates were affected by the heterobifunctional linkers used for connecting GLP-1C and CH (Figure 1B). The best overall yield of CH was approximately 20% and that of GLP-1C was around 60%. The low yield of CH was probably due to the limited content of amine-modified forms in the initial materials and subsequent multistep reactions.

Disaccharide Analysis of CH-Conjugated GLP-1C Peptides. The M_n of conjugated CH was calculated from the disaccharide analysis of CH-conjugated GLP-1 as follows: 6, 9, 11, 24, 34, 44, and 50 kDa for CH10 (n = 1), CH20 (n = 1), CH30 (n = 1), CH40 (n = 5), CH70 (n = 23), CH90 (n = 3), and CH140 (n = 21)-conjugated GLP-1C peptides, respectively. These values were slightly smaller (83% on average) than those of mother CHs (6 kDa for CH10, 11 kDa for CH20, 19 kDa for CH30, 26 kDa for CH40, 42 kDa for CH70, 49 kDa for CH90, and 67 kDa for CH140), respectively. This discrepancy is possibly due to overestimation of peptide concentrations because of the use of albumin as a control. Otherwise, a lower molecular weight population of CH might have reacted with better efficiency.

Structural Analysis of CH-Conjugated GLP-1C Peptides. To verify the structures of CH-conjugated peptides, they were treated with chondroitinase-ABC (C-ABC) and then with Glu-C endopeptidase. The C-ABC-digested products basically provided two major peaks (peaks 2 and 3) and two satellite peaks (peaks 1 and 4) (Figure 2A). Further Glu-C digestion of peaks 2 and 3 provided several peaks, of which the main peaks

DOI: 10.1021/acsomega.8b00467
ACS Omega 2018, 3, 5346−5354

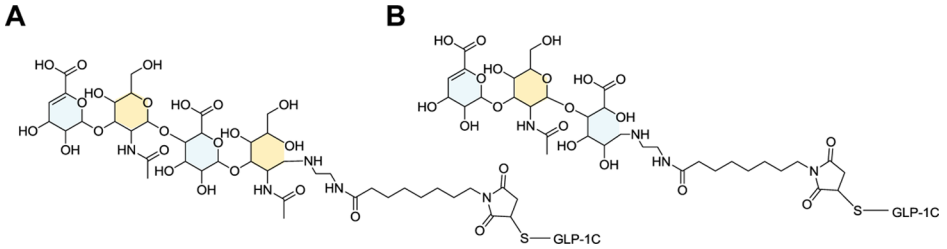


Figure 3. Structure of CH-conjugated GLP-1C peptides. The structures of peaks 2-C and 3-C identified by MS/MS are shown in (A,B), respectively.

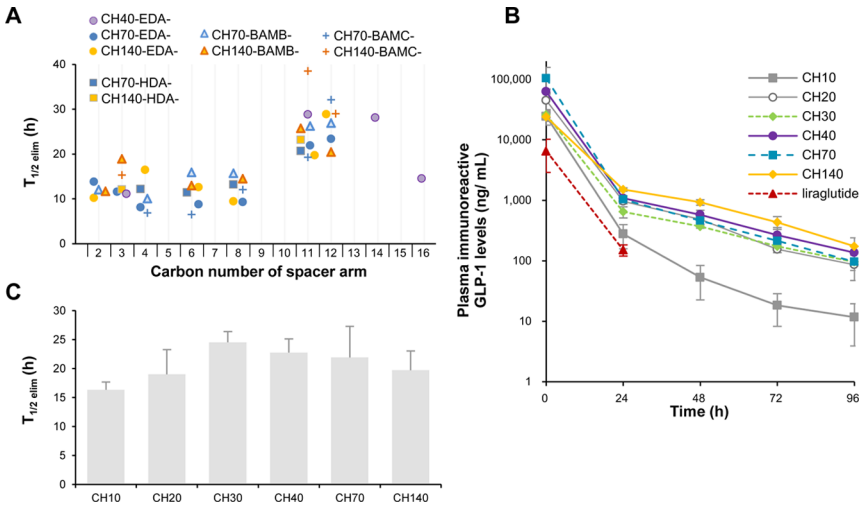


Figure 4. $T_{1/2\ elim}$ of CH-conjugated GLP-1C peptides administered to mice intravenously. (A) $T_{1/2\ elim}$ of CH40/CH70/CH140−EDA/HDA/BAMB/BAMC−(X)−GLP-1C was plotted vs the carbon number of the spacer arm of the heterobifunctional linker X: 2 for AMAS, 3 for BMPS, 4 for GMBS, 6 for EMCS, 8 for HMCS, 11 for KMUS, 12 for LMDS, 14 for NMTS, and 16 for OMHS. (B) Plasma immunoreactive GLP-1 levels after iv injection of CH10/CH20/CH30/CH40/CH70/CH140−EDA−(KMUS)−GLP-1C at 1 mg/kg as GLP-1C or liraglutide at 1 mg/kg. (C) $T_{1/2\ elim}$ of CH10/CH20/CH30/CH40/CH70/CH140−EDA−(KMUS)−GLP-1C conjugates containing various sizes of CH from the data shown in (B). All data in (B,C) are shown as means ± SD of triplicate determinations.

(2-C and 3-C) were found by MS/MS to be the C-terminal peptide (FIAWLVKGRC) conjugated with sugar moieties, respectively (Figure 2B). Peaks 2-C and 3-C were different in their sugar structure; the two peaks provided MS signals that matched peptides conjugated to GalNAc and GlcA, respectively (Figure 3). Additional MS/MS analyses of peaks 1 and 4 indicated that they were incompletely digested products elongated by a single disaccharide unit of peaks 2 and 3, respectively. These results indicated that CH preparation was heterogeneous in the sugar moiety of the reducing end.

GLP-1 Agonist Activity of CH-Conjugated GLP-1C Peptides. A variety of CH-conjugated GLP-1C peptides that differed in the heterobifunctional linkers used for conjugation, their molecular sizes, and their CH amino modifications were evaluated for their GLP-1 agonist activity by determining cyclic adenosine monophosphate (cAMP) production in RIN-m5F cells. CH-conjugated GLP-1C peptides showed EC_{50} values ranging from 5 to 20 nM, whereas unmodified GLP-1C peptide showed an EC_{50} value of 1 nM. There was no obvious relationship between the EC_{50} values and the linker structure of the conjugates. Liraglutide, an acylated analogue of GLP-1, showed the most potent agonist activity (EC_{50} = 0.7 nM). The PEG30k−GLP-1C peptide showed potent agonist activity (EC_{50} = 5.2 nM) compared to CH-conjugated GLP-1C.

Optimization of the Heterobifunctional Linker. Mice received iv injections of CH-conjugated GLP-1C peptide at a dose of 1 mg/kg as a GLP-1C peptide (ca. 300 nmol/kg), and the plasma concentration of conjugates was determined over time by using GLP-1 enzyme-linked immunosorbent assay (ELISA). All CH-conjugated peptides showed significantly prolonged half-lives (8.1−38.5 h) compared to that of GLP-1 (Figure 4A). Liraglutide (1 mg/kg) showed a moderate extent of retention in plasma, but far less than the CH-conjugated GLP-1C peptide (Figure 4C). The $T_{1/2\ elim}$ of liraglutide was unavailable (below the detection limit at 48 h). Notably, CH-conjugated GLP-1C peptides having a long alkyl chain derived from heterobifunctional linkers of *N*-[κ-maleimidoundecanoyloxy]-sulfosuccinimide ester (sulfo-KMUS, C11), *N*-[λ-maleimidododecanoyloxy]-sulfosuccinimide ester (sulfo-LMDS, C12), and *N*-[ν-maleimidotetradecanoyloxy]-sulfosuccinimide ester (sulfo-NMTS, C14) circulated for a longer period than those with short linkers (Figure 4A). However, CH-conjugated GLP-1C with long arms over C16 tended to show shortened $T_{1/2\ elim}$, indicating that a linker length of C11−C14 was optimum for conferring long blood circulation to the conjugate.

The relatively short optimum linker length suggests that the alkyl chains of CH-conjugated GLP-1C peptides are unlikely to serve in albumin binding, which is well-known for modifications with C16 or C18 chains.[11,30] Alternatively, we hypothesize that the alkyl chains interact with the hydrophobic surface of the GLP-1 α-helix to stabilize it. For exenatide, the C-terminal hydrophobic proline-rich sequence forms a compact structure (the Trp-cage), which surrounds and shields the side chain of the Trp[25] residue and stabilizes its α-helical structure.[10,31]

Optimization of the Molecular Weight of CH. The molecular weight of CH also affected the $T_{1/2\ elim}$ of CH-

DOI: 10.1021/acsomega.8b00467
ACS Omega 2018, 3, 5346−5354

conjugated peptides; the beneficial effect was relatively small ($T_{1/2\ elim}$ = 16 h) with CH10 (net bound CH of M_n = 6 kDa), most evident ($T_{1/2\ elim}$ = 22−25 h) with CH30, CH40, and CH70 (net bound CH of M_n = 11−35 kDa), and gradually decreased with increasing molecular weight (Figure 4C). However, there was no significant difference in plasma levels among these CH-conjugated peptides aside from CH10 (Figure 4B). Even CH10-conjugated peptide showed a fairly long circulation time compared to the PEG30k-conjugated GLP-1C peptide ($T_{1/2\ elim}$ = 17.5 h).

The result was much better than expected given that 10 kDa PEG-modified GLP-1 showed very short circulation time ($T_{1/2\ elim}$ = 105.5 min),[15] and the maximum effect was attained with tertiary-branched 23 kDa or 50 kDa PEG (39.6 and 51.2 h, respectively).[32] Therefore, we hypothesize that CH is more effective even at low molecular weight ranges than PEG in preventing renal clearance. The anionic charge and lower flexibility[33] of CH may have favored the slowing of glomerular filtration.[34] The slight decrease of $T_{1/2\ elim}$ at high molecular weight ranges may be related to easier capture of high molecular weight materials by phagocytotic Kupffer cells in the liver.[35,36]

PK of CH-Conjugated GLP-1C Peptide, Conjugate 1. Considering the agonist activity in vitro and the plasma circulation ($T_{1/2\ elim}$) after iv injection at 1 mg/kg, we selected CH70−EDA−(LMDS)−GLP-1C (conjugate **1**) for PK and pharmacodynamics (PD) studies, of which the EC_{50} and $T_{1/2\ elim}$ were 6.1 nM and 23.4 h, respectively. LMDS was the best for prolonging plasma circulation. EDA was the most efficient and stable for amine modification. CH70 and CH90 were the most convenient materials, requiring no additional downsizing procedures.

Conjugates were injected into Crl:CD1 (ICR) mice subcutaneously at 100 nmol/kg (0.33 mg/kg as GLP-1C peptide), and their plasma levels were determined. This is the highest dose usually used in rodent models according to the literature[37] and was thus appropriate for conjugate **1**, which had approximately 10 times less potent agonist activity than liraglutide.

As shown in Figure 5, **1** remained in circulation much longer ($T_{1/2\ elim}$ = 32.9 h) than liraglutide ($T_{1/2\ elim}$ = 8.9 h). The C_{max} values of **1** and liraglutide were 168.8 and 202.0 ng/mL, respectively. The T_{max} values were 24 and 6 h. The plasma levels of liraglutide increased and decreased more quickly after sc injection than those of **1**. The $AUC_{0-\infty}$ values were 8629.5 and 4084.3 ng/mL h. Mean residence times (MRT) were 44.8 and 15.5 h.

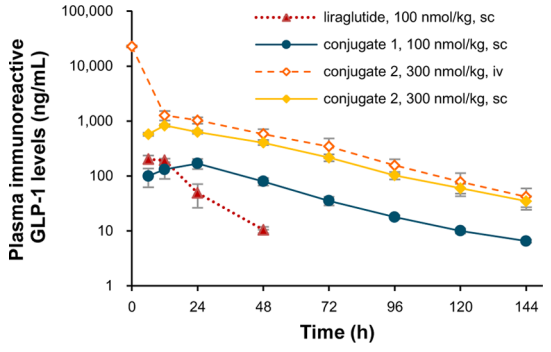


Figure 5. Plasma levels of CH-conjugated GLP-1C peptides. Plasma immunoreactive GLP-1 levels after sc injection of liraglutide and CH70−EDA−(LMDS)−GLP-1C (conjugate **1**) at 100 nmol/kg, and after sc and iv injections of CH90−EDA−(LMDS)−GLP-1C (conjugate **2**) at 300 nmol/kg as determined over time. All data are shown as means ± SD of triplicate determinations.

PD of CH-Conjugated GLP-1C Peptide, Conjugate 1. For PD studies, **1** and liraglutide were injected into ICR mice under the same conditions as for the PK studies, except for feeding conditions. After the administration of peptides, repeated cycles of intraperitoneal glucose-tolerance test (IPGTT) were performed every 24 h. In each IPGTT, blood glucose was determined at 0 min before glucose challenge and at 15, 30, and 60 min after glucose challenge. In the first cycle of IPGTT at 24 h postadministration, the increase of blood glucose levels was attenuated robustly by pretreatment with conjugated peptides (Figure 6A); however, this blood glucose-lowering effect was gradually blunted at 72 h (Figure 6C) and completely lost at 96 h (Figure 6D). On the basis of $AUC_{0-60min}$, **1** was effective until 72 h (Figure 6E) when its

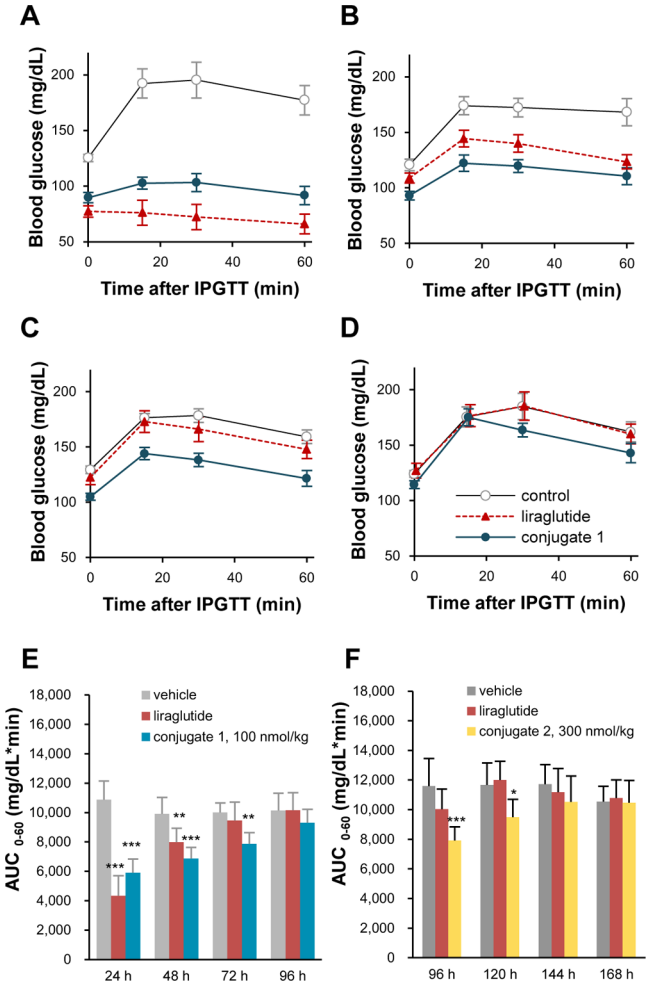


Figure 6. Blood glucose-lowering activity of CH-conjugated GLP-1C peptides in mice. IPGTT was performed at 24 (A), 48 (B), 72 (C), and 96 h (D) after sc injection of vehicle, liraglutide, or CH70−EDA−(LMDS)−GLP-1C (conjugate **1**) to mice at 100 nmol/kg. (E) $AUC_{0-60min}$ of blood glucose for (A−D) is shown. (F) $AUC_{0-60min}$ of IPGTT at indicated times after the sc injection of vehicle, liraglutide, or CH90−EDA−(LMDS)−GLP-1C (conjugate **2**) at 300 nmol/kg. Mean ± SD (n = 6). *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ vs vehicle (Dunnett's test).

DOI: 10.1021/acsomega.8b00467
ACS Omega 2018, 3, 5346−5354

plasma concentration was 35 ng/mL (Figure 5). This level is putatively the minimum effective concentration (MEC).

In contrast, liraglutide showed a very strong blood glucose-lowering effect in the first cycle of IPGTT (Figure 6A); it maintained blood glucose at mild hypoglycemic levels (around 70 mg/dL) even after glucose challenge. However, the efficacy of liraglutide was not durable and was swiftly lost at 72 h (Figure 6C,D). The temporal robustness of liraglutide's effect was likely due to the high agonist activity (EC_{50} = 0.7 nM) and steep increase of its plasma concentration.

PK and PD of CH-Conjugated GLP-1C Peptide, Conjugate 2. Next, PK studies were performed with CH90−EDA−(LMDS)−GLP-1C (conjugate **2**) at 300 nmol/kg (1 mg/kg as GLP-1C peptide), of which the chemical structure is the same as that of **1** except a small difference in the molecular weight of CH. Therefore, we assumed that **2** would be almost the same one as **1**. Indeed, the biological profile (EC_{50} = 9.9 nM, $T_{1/2\ elim}$ = 25.3 h after iv injection) of **2** was very similar to that of **1**. PK parameters were obtained from sc and iv injections (Figure 5) administered in parallel, and the bioavailability was obtained as 21% (Table 1). Dose escalation from 100 to 300 nmol/kg increased the plasma immunoreactive GLP-1 levels five times and delayed the levels reaching the putative MEC (35 ng/mL) by 3 days. Mice tolerated it well, and no remarkable adverse events were observed with any iv or sc injections, although the total mass of the conjugate became as high as 10−15 mg/kg at a dose of 300 nmol/kg.

Table 1. PK Parameters of CH− and HPN−EDA−(LMDS)−GLP-1C Conjugates[a]

	CH90−EDA−(LMDS)−GLP-1C (conjugate **2**)		HPN50−EDA−(LMDS)−GLP-1C (conjugate **3**)	
	iv injection	sc injection	iv injection	sc injection
$T_{1/2\ elim}$ (h)	25.3	30.3	33.6	25.8
T_{max} (h)	NA	12.0	NA	16.0
C_{max} (ng/mL)	22 704	828	63 287	1022
$AUC_{0-\infty}$ (ng/mL h)	199 809	42 954	497 318	61 370
V or V/F (mL/kg)	182	1016	103	599
Cl or Cl/F (mL/h/kg)	5.0	23.4	2.2	16.4
MRT (h)	14.1	46.0	15.3	51.0
BA (%)		21%		12%

[a]NA: not available.

IPGTT was performed at 96, 120, 144, and 168 h after dosing of **2** or liraglutide at 300 nmol/kg. The conjugated peptide **2** successfully displayed prolonged blood glucose-lowering efficacy until 120 h (Figure 6F); however, that was 1 day shorter than that expected from the plasma levels. The plasma levels of **2** at a dose of 300 nmol/kg were 60 ng/mL at 120 h and 40 ng/mL at 144 h (Figure 5), which were still higher than the putative MEC obtained from dosing at 100 nmol/kg. Therefore, we deduced that the MEC of **1** or **2** ranged between 35 and 60 ng/mL.

PK and PD of HPN-Conjugated GLP-1C Peptide, Conjugate 3. DeAngelis proposed that HPN is a promising conjugating polymer to extend the plasma residence of injectable therapeutics[38] and has demonstrated that 99 kDa HPN-conjugated G-CSF is very stable as shown by its $T_{1/2\ elim}$ of 8 days in a nonhuman primate.[39]

Because the linker design of **1** and **2** proved beneficial for improving the plasma circulation of CH-conjugated GLP-1C peptides, although we still wanted a more stable linker, we applied it to HPN50 (M_w/M_n = 51 kDa/32 kDa) to generate HPN50−EDA−(LMDS)−GLP-1C (conjugate **3**). Also, HPN50−EDA−(*N*-[*β*-Maleimidopropyloxy]succinimide ester (BMPS))−GLP-1C (conjugate **4**) with a short linker was prepared for comparison. After verifying their agonist activity in vitro (EC_{50} = 7.0 nM for **3** and 9.0 nM for **4**), these HPN-conjugated GLP-1C peptides were subjected to PK and PD studies by dosing at 300 nmol/kg.

Conjugate **3** with a long alkyl linker circulated more sustainably ($T_{1/2\ elim}$ = 33.6 h after iv injection) than **4** with a short linker ($T_{1/2\ elim}$ = 23.8 h) (Figure 7A), demonstrating that across different GAG species, the half-lives of GAG-conjugated GLP-1C peptides are affected by the length of the alkyl chain derived from the heterobifunctional linker. Furthermore, substitution of HPN for CH favorably affected the plasma circulation; HPN-conjugated GLP-1C showed better $T_{1/2\ elim}$ than that of CH-conjugated GLP-1C. Even HPN conjugate **4** with a short linker showed a $T_{1/2\ elim}$ over 20 h.

PK parameters from iv and sc injections of **3** are summarized in Table 1. Generally, **3** displayed better profiles than **2**. Because improvement of AUC by exchanging GAGs from CH to HPN was more prominent in iv injection than in sc injection, the bioavailability of **3** was decreased to 12%. The plasma concentration of **3** at 144 h after sc injection attained 62.9 ng/mL (Figure 7B), which was above the MEC of CH-conjugated GLP-1 (35−60 ng/mL). This indicated that the HPN-conjugated GLP-1C peptide, conjugate **3**, is more durable than the CH-conjugated GLP-1C peptide, conjugate **2**.

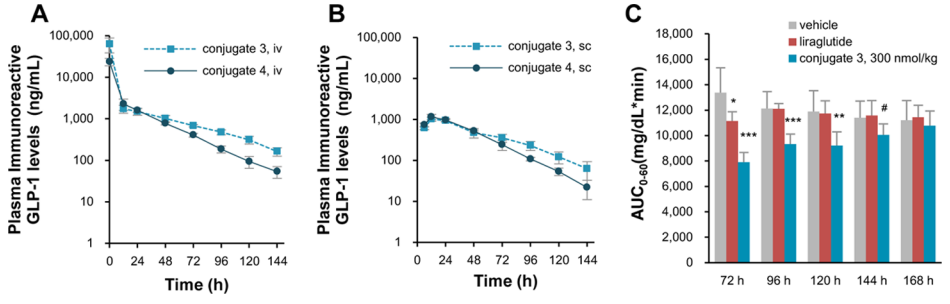


Figure 7. Plasma levels and blood glucose-lowering activity of HPN-conjugated GLP-1C in mice. Plasma immunoreactive GLP-1 levels after iv (A) and sc (B) injections of HPN50−EDA−(BMPS)−GLP-1C (conjugate **4**) and HPN50−EDA−(LMDS)−GLP-1C (conjugate **3**) at 300 nmol/kg. (C) $AUC_{0-60min}$ of IPGTT at indicated times after sc injection of vehicle, liraglutide, or HPN50−EDA−(LMDS)−GLP-1C at 300 nmol/kg. Mean ± SD (n = 6). #: $p < 0.1$, *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ vs vehicle (Dunnett's test).

DOI: 10.1021/acsomega.8b00467
ACS Omega 2018, 3, 5346−5354

Finally, HPN-conjugated peptide **3** was tested for its blood glucose-lowering activity at 300 nmol/kg administration. The conjugated peptide lowered glucose levels at 120 h after administration and still exerted activity at 144 h (Figure 7C). This is a favorable result in that conjugate **3** is approaching one-week duration, suggesting a need to compare it further with a weekly formulation, semaglutide.[11]

GAG conjugation has been shown to be successful in prolonging the circulation of medicinal peptides. The underlying mechanisms probably involve delayed renal clearance of conjugated GAG.[38] We have recently found that CH is long-lasting by itself in mice, whereas CH sulfate and hyaluronan disappear very shortly.[40] Also, isolation from the reticuloendothelial system and plasma proteases (i.e., molecular shielding) is another possible mechanism not specifically addressed in this study. Nevertheless, the circulating GLP-1 immunoreactivity probably reflected uncleaved peptides because of the specificity of the enzyme immunoassay and the prolonged duration of efficacy. This suggests that GAG modification adequately protected GLP-1C molecules. Molecular shielding can also cause steric hindrance to receptor interactions. In this aspect, the agonist activity of GAG-conjugated GLP-1C peptides was roughly in the same range as that of PEGylated GLP-1.

It has yet to be revealed which mechanisms make particular kinds of GAG long-lasting in mouse circulation and how the underlying mechanisms are conserved through the species. Regarding safety, we preliminarily found that CH circulates in humans and that consecutive iv injections of CH in rats at 100 mg/kg for 28 days did not cause any adverse events or histological changes such as vacuolation, suggesting that CH is an inert material.[29,40] The safety of CH and HPN is a critical subject to be carefully investigated in the future. In conclusion, our results suggest the use of GAG modification as an option to improve the performance of medicinal peptides.

EXPERIMENTAL PROCEDURES

Cross-linkers. *N*-[α-Maleimidoacetoxy]succinimide ester (AMAS) was purchased from Thermo Fisher Scientific (Waltham, MA), and EDA was purchased from Wako Pure Chemical Industries (Osaka, Japan). BMPS, HDA, BAMC (cis- and trans-mixture), and BAMB were from Tokyo Chemical Industry (Tokyo, Japan). *N*-[γ-Maleimidobutyryloxy]-sulfosuccinimide ester (sulfo-GMBS), *N*-[ε-maleimidocaproyloxy]sulfosuccinimide ester (sulfo-EMCS), *N*-(η-maleimidocapryloxy)sulfosuccinimide (sulfo-HMCS), and sulfo-KMUS were from Dojindo Laboratories (Kumamoto, Japan). sulfo-LMDS, sulfo-NMTS, and *N*-[o-maleimidohexadecanoyloxy]-sulfosuccinimide ester (sulfo-OMHS) were synthesized in our laboratory.

Peptides. GLP-1C (HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRC) was purchased from Scrum Inc. (Tokyo, Japan). Liraglutide (Victoza) was obtained from Novo Nordisk A/S (Bagsværd, Denmark).

Glycosaminoglycans. CH was produced by elongation from glycolipid initiators via the capsular polysaccharide biosynthetic pathway in gene-engineered *Escherichia coli* (MSC702 strain).[41] For the preparation of CH70, CH90, and CH140, mild alkaline treatment was exploited both for removing the glycolipid moiety and decreasing the molecular size by sequential degradation of the polysaccharide chains. Additional acid treatment was used to further downsize CH70 (or CH90) into CH10, CH20, CH30, and CH40.[42] Purification was principally done by alcohol precipitation. Treatment with acetic anhydride was done to secure the GalNAc structure. It was inevitable that a relatively high degree of polydispersity was found for the molecular weight of CH as follows: CH10 (M_w/M_n = 10 kDa/6 kDa), CH20 (21 kDa/11 kDa), CH30 (31 kDa/19 kDa), CH40 (43 kDa/26 kDa), CH70 (69 kDa/42 kDa), CH90 (92 kDa/49 kDa), and CH140 (141 kDa/67 kDa). Polydispersity (M_w/M_n) was around 1.6−2.1. HPN50 (M_w/M_n = 51 kDa/32 kDa) was purified from the fermentation broth of the K5 strain of *E. coli* in the same manner as CH.

Reductive Amination of GAGs. GAGs (CH and HPN) were modified to amine forms by the reductive amination of aldehyde groups with diamine (aliphatic or aromatic) using an excess amount of the reducing agent sodium cyanoborohydride (Sigma-Aldrich, St. Louis, MO) or 2-picoline borane (Tokyo Chemical Industry). After the reaction, GAGs were purified by repeated cycles of ethanol precipitation followed by gel filtration in 0.1 M ammonium formate buffer through two tandem-connected 26/10 desalting columns (GE Healthcare, Chicago, IL) equipped with AKTA avant 25 (GE Healthcare) followed by exhaustive dialysis against distilled water (DW). The purified GAGs were lyophilized, and the content of amino groups was determined by colorimetric quantification with *o*-phthalaldehyde.[43]

Preparation of GAG-Conjugated GLP-1C Peptides. Amine-modified GAGs (20 mg) were dissolved in 50% dimethylformamide (DMF) at 40 mg/mL, mixed with 10 mM heterobifunctional linkers (120 μL) and 0.5 M bicine-HCl buffer (pH 8.3, 80 μL), and reacted for 1 h at room temperature in the dark. The reaction mixture was diluted to 3.2 mL with 0.1 M ammonium formate buffer, and aliquots (1.6 mL) were subjected to gel-filtration chromatography on seven tandem-connected HiTrap Desalting columns (GE Healthcare) in 0.1 M ammonium formate buffer at a flow rate of 3 mL/min.

The purified GAG fraction was lyophilized, dissolved in DW (650 μL), and mixed with 0.1 mg/mL GLP-1C/DMF (80 μL), acetonitrile (325 μL), and 0.5 M HEPES buffer (pH 7.0, 80 μL), and reacted for 16 h at room temperature in the dark. The reaction mixture was diluted with 0.1% trifluoroacetic acid (TFA)/DW (6 mL), centrifuged at 20 600*g* for 10 min, and injected into a reversed-phase HPLC column (DAISO, SP-120-5-ODS-BP, 20 mm × 250 mm) equipped with a Waters 600 series pump (Waters Corp., Milford, MA) at a flow rate of 8 mL/min at 40 °C. Then, the GAG-conjugated peptide was eluted by a linear gradient increase of acetonitrile concentration from 8 to 48% for 30 min. PEGylated GLP-1C was also prepared by reacting GLP-1C with PEG30k−maleimide (SUNBRIGHT ME-300MA, NOF, Tokyo, Japan). Purification was performed by reversed-phase HPLC.

The purified conjugated peptide was lyophilized, weighed, dissolved in DW at a concentration of 4 mg powder/mL, and subjected to bicinchoninic acid protein assay (Thermo Fisher Scientific). The calibration curve was generated by using bovine serum albumin standard (Thermo Fisher Scientific), although it overestimated peptide concentration up to 1.5-fold compared to that made with a GLP-1 standard (Peptide Institute, Osaka, Japan). The weights of conjugated peptide and liraglutide are shown by GLP-1 peptide-equivalent weights and not by the whole weight of conjugated peptide or liraglutide.

Quantification of CH. Disaccharide analysis was performed for CH-conjugated peptides to quantify the content of CH. Briefly, the conjugated peptide was digested with 0.125 U/12.5 μL C-ABC (Seikagaku Corp.) and 0.0125 U/12.5 μL CHase ACII (ACII, Seikagaku Corp.) at 37 °C for 2 h and then

DOI: 10.1021/acsomega.8b00467
ACS Omega 2018, 3, 5346−5354

subjected to HPLC analysis with monitoring by postcolumn labeling as described previously.[44] The number of total disaccharide units and the whole molecular mass of CH were calculated per peptide molecule.

Structural Analysis of CH-Conjugated GLP-1C Peptides. CH-conjugated GLP-1 peptides were dissolved in DW (100 μL) at 0.3−0.5 mg/mL, mixed with 0.4 M Tris-HCl (pH 8.0, 50 μL), 0.4 M sodium acetate (50 μL), DW (400 μL), and 10 U/mL C-ABC (50 μL), and incubated at 37 °C for 2 h. Digested peptide was boiled, diluted with 0.1% TFA, injected into a reversed-phase HPLC column (DAISO SP120-5-ODS-BP, 4.6 × 150 mm), and eluted by a linear gradient increase of acetonitrile concentration from 28 to 48% over 30 min at a flow rate of 0.83 mL/min. The peak fractions were obtained and lyophilized. Next, the lyophilized peptide was dissolved in 0.1 M acetate ammonium (50 μL) and digested with Glu-C endopeptidase (Thermo Fisher Scientific, 1.9 μL) at 37 °C overnight. The digested peptide was injected into a reversed-phase HPLC column (DAISO SP120-5-ODS-BP, 4.6 × 150 mm) and eluted by a linear gradient increase of acetonitrile concentration from 0 to 100% over 30 min at a flow rate of 0.83 mL/min. The peak fractions were analyzed by amaZon speed ETD (Bruker, Billerica, MA).

Assay of GLP-1 Agonist Activities. RIN-m5F cells (ATCC, Manassas, VA) were cultured at a seeding density of 40 000 cells/well in 10% fetal calf serum/RPMI 1640 medium (both from Thermo Fisher Scientific) in BIOCOAT poly-D-lysine-coated, white-wall 96-well plates (Becton Dickinson, Franklin Lakes, NJ) for 16 h at 37 °C under 5% CO_2. The cells were starved in a serum-free RPMI 1640 medium for 6 h, treated with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and 0.1 mM Ro-20-1724 (Sigma-Aldrich) for 15 min, and then challenged with varying concentrations of GLP-1C or CH-conjugated GLP-1C peptides for 10 min. Then, cAMP levels were determined in situ using a cAMP-Glo Max Assay kit (Promega, Fitchburg, WI). Luminescence intensity was measured with an ARVO ×5 plate reader (PerkinElmer, Waltham, MA).

Animals. Male ICR mice were obtained from Charles River Laboratories (Yokohama, Japan) at 5 weeks old and acclimatized for 5−10 days in the specific pathogen-free animal facility under a 12 h light/12 h dark cycle, and controlled temperature (20−26 °C), and humidity (30−70%). All mice were given free access to chow diet (except during glucose-tolerance tests) and water. All procedures for animal care and animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Seikagaku Corporation.

PK Studies. Male ICR mice (6 weeks old, $n = 3$) received iv injections of GAG-conjugated GLP-1C at 1 mg/kg, as GLP-1C peptide or liraglutide at 1 mg/kg, in a screening study. To compare PK and PD, the mice received iv injections of GAG-conjugated GLP-1C at 300 nmol/kg, sc injections of GAG-conjugated GLP-1C at 100 or 300 nmol/kg, or sc injection of liraglutide at 100 nmol/kg. Blood samples were collected from the tail vein at 0, 12, 24, 48, 72, 96, 120, and 144 h postadministration. Plasma samples were prepared from the blood samples and stored at −80 °C until use. The concentration of immunoreactive GLP-1 in the plasma samples was quantified using a glucagon-like peptide-1 (active) ELISA kit (Millipore, Darmstadt, Germany). The fluorescence intensity was measured with a microplate reader (ARVO ×5, PerkinElmer). The standard curve was generated by fitting the four parameters to a logistic regression using Origin ver. 9.1 (OriginLab Corp., Northampton, MA). Plasma half-life time was calculated using Phoenix WinNonlin Version 6.4 (Pharsight Corp., St. Louis, MO), and mean values of three measurements were obtained.

IPGTT for PD. Male ICR mice (6 weeks old, $n = 6$) received sc injections of GAG-conjugated GLP-1C or liraglutide at 100 and 300 nmol/kg or with saline for control. At 24, 48, 72, and 96 h after dosing at 100 nmol/kg or at 96, 120, 144, and 168 h after dosing at 300 nmol/kg, the mice were challenged with intraperitoneal injection of glucose at 1 g/kg preceded by food deprivation for 6 h and blood glucose levels were measured at 0 min before glucose challenge and 15, 30, and 60 min after glucose challenge under food-deprived conditions. Mice were re-fed and rested for the next IPGTT. A self-monitoring blood glucose meter (Eisai, Tokyo, Japan) was used to measure glucose levels in blood samples (5 μL). The blood glucose levels were plotted over time, and the $AUC_{0-60min}$ was obtained. Statistical analysis was by Dunnett's test.

AUTHOR INFORMATION

Corresponding Author

*E-mail: yoshihiro.takatsu@seikagaku.co.jp. Phone: +81-3-5220-8559. Fax: +81-3-5220-8594 (Y.T.).

ORCID

Megumi Ichikawa: 0000-0001-8916-0251

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Kiyoshi Suzuki, Kiwamu Yamagishi, Kazuhiko Tsujita, and Makoto Takashima for providing CH and HPN and to Drs. Masaomi Miyamoto and Hiroshi Maeda for their helpful discussions and encouragement throughout this work.

REFERENCES

(1) Deacon, C. F. Therapeutic Strategies Based on Glucagon-Like Peptide 1. *Diabetes* **2004**, *53*, 2181−2189.

(2) Drucker, D. J.; Nauck, M. A. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* **2006**, *368*, 1696−1705.

(3) Holst, J. J. Glucagon-like peptide-1: from extract to agent. The Claude Bernard Lecture, 2005. *Diabetologia* **2006**, *49*, 253−260.

(4) Meier, J. J.; Nauck, M. A. Glucagon-like peptide 1(GLP-1) in biology and pathology. *Diabetes/Metab. Res. Rev.* **2005**, *21*, 91−117.

(5) Holst, J. J. The Physiology of Glucagon-like Peptide 1. *Physiol. Rev.* **2007**, *87*, 1409−1439.

(6) Vilsbøll, T.; Agersø, H.; Krarup, T.; Holst, J. J. Similar Elimination Rates of Glucagon-Like Peptide-1 in Obese Type 2 Diabetic Patients and Healthy Subjects. *J. Clin. Endocrinol. Metab.* **2003**, *88*, 220−224.

(7) Deacon, C. F.; Knudsen, L. B.; Madsen, K.; Wiberg, F. C.; Jacobsen, O.; Holst, J. J. Dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-1 which have extended metabolic stability and improved biological activity. *Diabetologia* **1998**, *41*, 271−278.

(8) Mentlein, R.; Gallwitz, B.; Schmidt, W. E. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur. J. Biochem.* **1993**, *214*, 829−835.

(9) Jelsing, J.; Vrang, N.; Hansen, G.; Raun, K.; Tang-Christensen, M.; Knudsen, L. B. Liraglutide: short-lived effect on gastric emptying-

DOI: 10.1021/acsomega.8b00467
ACS Omega 2018, 3, 5346−5354

long lasting effects on body weight. *Diabetes, Obes. Metab.* **2012**, *14*, 531−538.
(10) Lorenz, M.; Evers, A.; Wagner, M. Recent progress and future options in the development of GLP-1 receptor agonists for the treatment of diabesity. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 4011−4018.
(11) Lau, J.; Bloch, P.; Schäffer, L.; Pettersson, I.; Spetzler, J.; Kofoed, J.; Madsen, K.; Knudsen, L. B.; McGuire, J.; Steensgaard, D. B.; Strauss, H. M.; Gram, D. X.; Knudsen, S. M.; Nielsen, F. S.; Thygesen, P.; Reedtz-Runge, S.; Kruse, T. Discovery of the Once-Weekly Glucagon-Like Peptide-1 (GLP-1) Analogue Semaglutide. *J. Med. Chem.* **2015**, *58*, 7370−7380.
(12) Lee, S.-H.; Lee, S.; Youn, Y. S.; Na, D. H.; Chae, S. Y.; Byun, Y.; Lee, K. C. Synthesis, characterization, and pharmacokinetic studies of PEGylated glucagon-like peptide-1. *Bioconjugate Chem.* **2005**, *16*, 377−382.
(13) Gao, M.; Tong, Y.; Gao, X.; Yao, W. Development of a C-Terminal Site-Specific PEGylated Analog of GLP-1 with Improved Anti-Diabetic Effects in Diabetic Mice. *Drug Dev. Res.* **2013**, *74*, 186−193.
(14) Lee, S.; Youn, Y. S.; Lee, S.-H.; Byun, Y.; Lee, K. C. PEGylated glucagon-like peptide-1 displays preserved effects on insulin release in isolated pancreatic islets and improved biological activity in db/db mice. *Diabetologia* **2006**, *49*, 1608−1611.
(15) Chae, S. Y.; Chun, Y. G.; Lee, S.; Jin, C.-H.; Lee, E. S.; Lee, K. C.; Youn, Y. S. Pharmacokinetic and pharmacodynamic evaluation of site-specific PEGylated glucagon-like peptide-1 analogs as flexible postprandial-glucose controllers. *J. Pharm. Sci.* **2009**, *98*, 1556−1567.
(16) Zhang, F.; Liu, M.-r.; Wan, H.-t. Discussion about several potential drawbacks of PEGylated therapeutic proteins. *Biol. Pharm. Bull.* **2014**, *37*, 335−339.
(17) Bendele, A. Short Communication: Renal Tubular Vacuolation in Animals Treated with Polyethylene-Glycol-Conjugated Proteins. *Toxicol. Sci.* **1998**, *42*, 152−157.
(18) Rudmann, D. G.; Alston, J. T.; Hanson, J. C.; Heidel, S. High Molecular Weight Polyethylene Glycol Cellular Distribution and PEG-associated Cytoplasmic Vacuolation Is Molecular Weight Dependent and Does Not Require Conjugation to Proteins. *Toxicol. Pathol.* **2013**, *41*, 970−983.
(19) Hershfield, M. S.; Ganson, N. J.; Kelly, S. J.; Scarlett, E. L.; Jaggers, D. A.; Sundy, J. S. Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for refractory gout, including in organ transplant recipients. *Arthritis Res. Ther.* **2014**, *16*, R63.
(20) Sattui, S. E.; Gaffo, A. L. Treatment of hyperuricemia in gout: current therapeutic options, latest developments and clinical implications. *Ther. Adv. Musculoskeletal Dis.* **2016**, *8*, 145−159.
(21) Berhanu, A. A.; Krasnokutsky, S.; Keenan, R. T.; Pillinger, M. H. Pegloticase failure and a possible solution: Immunosuppression to prevent intolerance and inefficacy in patients with gout. *Semin. Arthritis Rheum.* **2017**, *46*, 754−758.
(22) Garay, R. P.; El-Gewely, R.; Armstrong, J. K.; Garratty, G.; Richette, P. Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. *Expert Opin. Drug Delivery* **2012**, *9*, 1319−1323.
(23) Ganson, N. J.; Povsic, T. J.; Sullenger, B. A.; Alexander, J. H.; Zelenkofske, S. L.; Sailstad, J. M.; Rusconi, C. P.; Hershfield, M. S. Pre-existing anti-polyethylene glycol antibody linked to first-exposure allergic reactions to pegnivacogin, a PEGylated RNA aptamer. *J. Allergy Clin. Immunol.* **2016**, *137*, 1610−1613.e7.
(24) Pelegri-O'Day, E. M.; Lin, E.-W.; Maynard, H. D. Therapeutic Protein-Polymer Conjugates: Advancing Beyond PEGylation. *J. Am. Chem. Soc.* **2014**, *136*, 14323−14332.
(25) Liebner, R.; Mathaes, R.; Meyer, M.; Hey, T.; Winter, G.; Besheer, A. Protein HESylation for half-life extension: Synthesis, characterization and pharmacokinetics of HESylated anakinra. *Eur. J. Pharm. Biopharm.* **2014**, *87*, 378−385.
(26) Gregoriadis, G.; Jain, S.; Papaioannou, I.; Laing, P. Improving the therapeutic efficacy of peptides and proteins: A role for polysialic acids. *Int. J. Pharm.* **2005**, *300*, 125−130.
(27) Jain, S.; Hreczuk-Hirst, D. H.; McCormack, B.; Mital, M.; Epenetos, A.; Laing, P.; Gregoriadis, G. Polysialylated insulin: synthesis, characterization and biological activity in vivo. *Biochim. Biophys. Acta, Gen. Subj.* **2003**, *1622*, 42−49.
(28) Chen, C.; Constantinou, A.; Chester, K. A.; Vyas, B.; Canis, K.; Haslam, S. M.; Dell, A.; Epenetos, A. A.; Deonarain, M. P. Glycoengineering Approach to Half-Life Extension of Recombinant Biotherapeutics. *Bioconjugate Chem.* **2012**, *23*, 1524−1533.
(29) Takatsu, Y. Chonylation: A new drug delivery technology for improving the therapeutic efficacy of proteins and peptides utilizing naturally-occurring sugar polymer chondroitin (CH) as an alternative to polyethylene glycol. *J. Pharm. Drug Deliv. Res.* **2017**, *6*, 90.
(30) Madsen, K.; Knudsen, L. B.; Agersoe, H.; Nielsen, P. F.; Thøgersen, H.; Wilken, M.; Johansen, N. L. Structure−Activity and Protraction Relationship of Long-Acting Glucagon-like Peptide-1 Derivatives: Importance of Fatty Acid Length, Polarity, and Bulkiness. *J. Med. Chem.* **2007**, *50*, 6126−6132.
(31) Neidigh, J. W.; Fesinmeyer, R. M.; Prickett, K. S.; Andersen, N. H. Exendin-4 and Glucagon-like-peptide-1: NMR Structural Comparisons in the Solution and Micelle-Associated States. *Biochemistry* **2001**, *40*, 13188−13200.
(32) Kim, T. H.; Jiang, H. H.; Lim, S. M.; Youn, Y. S.; Choi, K. Y.; Lee, S.; Chen, X.; Byun, Y.; Lee, K. C. Site-Specific PEGylated Exendin-4 Modified with a High Molecular Weight Trimeric PEG Reduces Steric Hindrance and Increases Type 2 Antidiabetic Therapeutic Effects. *Bioconjugate Chem.* **2012**, *23*, 2214−2220.
(33) Almond, A.; Sheehan, J. K. Glycosaminoglycan conformation: do aqueous molecular dynamics simulations agree with x-ray fiber diffraction? *Glycobiology* **2000**, *10*, 329−338.
(34) Thomson, S. C.; Blantz, R. C. *Biophysics of Glomerular Filtration, in Comprehensive Physiology*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2012; pp 1671−1699.
(35) Yamaoka, T.; Tabata, Y.; Ikada, Y. Distribution and Tissue Uptake of Poly(ethylene glycol) with Different Molecular Weights after Intravenous Administration to Mice. *J. Pharm. Sci.* **1994**, *83*, 601−606.
(36) Fishburn, C. S. The Pharmacology of PEGylation: Balancing PD with PK to Generate Novel Therapeutics. *J. Pharm. Sci.* **2008**, *97*, 4167−4183.
(37) Rolin, B.; Larsen, M. O.; Gotfredsen, C. F.; Deacon, C. F.; Carr, R. D.; Wilken, M.; Knudsen, L. B. The long-acting GLP-1 derivative NN2211 ameliorates glycemia and increases β-cell mass in diabetic mice. *Am. J. Physiol. Endocrinol. Metab.* **2002**, *283*, E745−E752.
(38) DeAngelis, P. L. Heparosan, a promising "naturally good" polymeric conjugating vehicle for delivery of injectable therapeutics. *Expert Opin. Drug Delivery* **2015**, *12*, 349−352.
(39) Jing, W.; Roberts, J. W.; Green, D. E.; Almond, A.; DeAngelis, P. L. Synthesis and characterization of heparosan-granulocyte-colony stimulating factor conjugates: a natural sugar-based drug delivery system to treat neutropenia. *Glycobiology* **2017**, *27*, 1052−1061.
(40) Fukushima, M. Chondroitin Is a Favorable Bio-polymer for Manufacturing of Long-Acting Diabetic Therapeutics. *American Diabetes Association 77th Scientific Sessions*, 2017.
(41) Doherty, D. H., Weaver, C. A., Miyamoto, K., Minamisawa, T. Compositions and methods for bacterial and genetically modified microorganism production of chondroitin. US Patent 9,175,293 B2, (accessed October 21, 2017).
(42) Stern, R.; Kogan, G.; Jedrzejas, M. J.; Šoltés, L. The many ways to cleave hyaluronan. *Biotechnol. Adv.* **2007**, *25*, 537−557.
(43) Benson, J. R.; Hare, P. E. o-Phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 619−622.
(44) Toyoda, H.; Shinomiya, K.; Yamanashi, S.; Koshiishi, I.; Imanari, T. Microdetermination of Unsaturated Disaccharides Produced from Chondroitin Sulfates in Rabbit Plasma by High Performance Liquid Chromatography with Fluorometric Detection. *Anal. Sci.* **1988**, *4*, 381−384.

DOI: 10.1021/acsomega.8b00467
ACS Omega 2018, 3, 5346−5354

NOTE ADDED IN PROOF

Part of the data was previously published as a poster at the Controlled Research Society Meeting 2017 and at the American Diabetes Meeting 2017.

DOI: 10.1021/acsomega.8b00467
ACS Omega 2018, 3, 5346−5354