



N-Glycosylation and enzymatic activity of the rHuPH20 expressed in Chinese hamster ovary cells

Tao Liu^{a,b,c,1}, Yantao Li^{b,c,1}, Jin Xu^{b,c,d,e,1}, Qingcheng Guo^{b,c,d,g}, Dapeng Zhang^{b,c,d}, Lankun Song^f, Jun Li^d, Weizhu Qian^{b,c,d}, Huaizu Guo^{b,c,d,e,***}, Xinli Zhou^{a,**}, Sheng Hou^{b,c,d,*}

^a Department of Oncology, Huashan Hospital, Fudan University, Shanghai, 200043, China

^b State Key Laboratory of Antibody Medicine and Targeted Therapy, Shanghai, 201203, China

^c NMPA Key Laboratory for Quality Control of Therapeutic Monoclonal Antibodies, Shanghai, 201203, China

^d School of Pharmacy, Liaocheng University, Liaocheng, 252000, China

^e Shanghai Zhangjiang Biotechnology Co., Ltd, Shanghai, 201203, China

^f Waters Corporation, Shanghai, 201206, China

^g Taizhou Mabtech Pharmaceuticals Co., Ltd, Taizhou 225316, China

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ABSTRACT

rHuPH20, a neutral pH-active hyaluronidase that degrades glycosaminoglycans under physiologic conditions, has six potential N-glycosylation sites. In this report, the rHuPH20 expressed in Chinese hamster ovary (CHO) cells was analyzed and characterized using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Removal of the N-linked glycans from rHuPH20 with PNGase F shifted the molecular weight from 66 kDa to approximately 52 kDa, its deduced molecular weight based on sequence analysis, suggesting that most, if not all, of the potential N-glycosylation sites are linked to oligosaccharides. Then the N-linked glycans released from the rHuPH20 by PNGase F were characterized by UPLC-FLR-MS, and the six N-glycosylation sites of the rHuPH20 were identified and characterized by UPLC-MS/MS at peptide levels. Subsequently, we found that the rHuPH20 increased the dispersion of locally subcutaneous injected drugs and the *in vitro* and *in vivo* bioactivity were decreased significantly after PNGase F treatment. In particular, rHuPH20 significantly augmented the absolute bioavailability of locally subcutaneous injected large protein therapeutics, while the bioavailability decreased after being digested by PNGase F. These results demonstrated that N-glycosylation is important for the bioactivity of the rHuPH20.

1. Introduction

PH20 is a member of the hyaluronidase family, which hydrolyzes the β -1-4 linkage between N-acetylglucosamine and glucuronic acid of hyaluronic acid (HA) polymers furnishing tetrasaccharide molecule as the main product [1,2]. HA, which is an anionic, non-sulfated glycosaminoglycan that exists as a megadalton polymer of disaccharides, is a major component of the extracellular matrix. The disaccharide is composed of D-glucuronic acid and D-N-acetylglucosamine that are linked via alternating β -1,4 and β -1,3 glycosidic bonds. The PH20 is

glycosidase with both hydrolytic and transglycosidase activity and degrades HA, chondroitin, chondroitin-4,6-sulphate to a small extent, dermatan sulphate [3,4]. The human genome contains six hyaluronidase-like genes [5,6]. The sequences of the six subtypes of human hyaluronidases have 33–44% similarity [7]. PH20 is the predominant hyaluronidase for degrading human HA in mammalian testes [8]. In the human body, PH20 is mainly expressed in testis, however, it can also be detected in the epididymis, female genital tract, breast, placenta fetal tissues, synoviocytes and skin fibroblast cell lines by sensitive techniques [9]. Among the six human hyaluronidases-like

Abbreviations: rHuPH20, recombinant human PH20; UPLC, Ultra-performance liquid chromatography; Q-ToF, quadrupole-time of flight; MS, mass spectrometry; ESI, electrospray ionization; IAM, Iodoacetamide; DTT, dithiothreitol; FA, formic acid; PTMs, post-translational modifications; TIC, Total Ion Chromatography.

* Corresponding author. State Key Laboratory of Antibody Medicine and Targeted Therapy, Shanghai, 201203, China.

** Corresponding author. Department of Oncology, Huashan Hospital, Fudan University, Shanghai, 200043, China.

*** Corresponding author. State Key Laboratory of Antibody Medicine and Targeted Therapy, Shanghai, 201203, China.

E-mail addresses: guohuaizu@163.com (H. Guo), xinli_zhou73@163.com (X. Zhou), housheng301@163.com (S. Hou).

¹ These authors contributed equally to this work.

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genes, only the PH20 gene product is a neutral as well as acidic pH-active hyaluronidase and degrades glycosaminoglycans under physiologic conditions [10,11].

PH20 is a testicular hyaluronidase that was found as early as 1983 in guinea pig sperm and exists widely in the mammalian sperm [12]. The major role of PH20 is to facilitate penetration of the sperm through the HA-rich matrix of the oocyte [1,13]. Animal-derived hyaluronidase from testes extracts has been used as an interstitial delivery enhancing agent to facilitate the dispersion and absorption of other drugs for over 60 years [14]. Furthermore, hyaluronidase is also used in ophthalmologic surgery [15]. Tumors that appear to be resistant to chemotherapy, can also be rendered sensitive when the mammalian sperm-derived PH20 is added [16]. However, they have generally been limited by both immunogenicity and impurity profiles which increase capillary permeability and give rise to IgE mediated allergic reactions upon repeat administration, generally precluding their repeated use [17,18]. In order to avoid or to reduce the risk of allergic reaction which is caused by the impure hyaluronidase injection, we generated a highly purified recombinant human hyaluronidase (rHuPH20) was produced in Chinese hamster ovary (CHO) cells. The human PH20 is a 509 amino acid glycoprotein anchored to the plasma membrane of mammalian sperm heads through a glycosyl-phosphatidylinositol (GPI) moiety that facilitates penetration of sperm through the cumulus cells to enable fertilization [11]. However, the rHuPH20 is a 447 amino acid 61 kDa glycoprotein with a properly processed amino terminus lacking the GPI membrane attachment motif [19].

Glycosylation plays an important role in stability, *in vivo* activity, serum half-life, and immunogenicity of many recombinant therapeutic proteins [20]. The roles of glycosylation in pharmacokinetics, bioavailability, clearance, and potency are demonstrated by preclinical and clinical studies from which the acceptable variability is also defined [21]. N-glycosylation is a well-known type of glycosylation in which N-glycans are directly attached to asparagine residue in proteins [22,23]. The aberrant condition of N-glycosylation is known to correlate with many diseases, such as cancer and autoimmune diseases [24,25]. Furthermore, in humans, a defect in N-glycosylation induces disorders, which are often referred to as congenital disorders of glycosylation [26]. Therefore, further detailed investigations are required to clarify the roles of the modification. Analysis of the N-glycans attached to therapeutic proteins, on the other hand, provides crucial information on the types of glycan structures that exist among the proteins, helps to define the heterogeneity of the sample. Information acquired from the analysis of protein glycosylation also helps to establish structure-function relationships for protein therapeutics.

PH20 is known to have a number of potential N-glycosylation sites characterized by the amino acid sequence Asp-X_{AA}-Ser/Thr, in which the internal X is not a proline, although this sequence is not always glycosylated in the PH20 of different mammalian species [27]. Oligosaccharide structures attached to proteins is conserved in eukaryotes. The rHuPH20 has six potential N-linked glycosylation sites. However, there has been no direct evidence that demonstrates the presence of N-glycosylation within rHuPH20 by mass spectrometry. We therefore undertook quadrupole time of flight mass spectrometry (Q-TOF-MS) analysis to examine whether rHuPH20 is N-glycosylated and whether all the potential N-glycosylation sites are located. Furthermore, the importance of N-glycosylation of rHuPH20 on enzymatic activity was demonstrated.

In this study, we examined the N-glycosylation of rHuPH20 expressed in CHO cells and determined its functional significance. The N-linked glycans released by PNGase F were characterized by UPLC-FLR-MS. The N-glycosylation sites were further identified and characterized by UPLC-MS/MS at peptide levels. Subsequently, we found that the rHuPH20 increased the dispersion of locally subcutaneous injected drugs and the *in vitro* and *in vivo* bioactivity were decreased significantly after PNGase F treatment. We could demonstrate that the six predicted asparagine residues were N-glycosylated and the glycosylation appeared

to play an important role in the enzymatic activity of rHuPH20. we found that the rHuPH20 increased the dispersion of locally subcutaneous injected drugs and the *in vitro* and *in vivo* bioactivity were decreased significantly after PNGase F treatment. This study proved that N-glycosylation of the rHuPH20 is necessary for its bioactivity.

2. Materials and methods

2.1. Materials

The atezolizumab biosimilar candidate and rHuPH20 were manufactured in our lab using CHO cells, purified with sequential chromatographic techniques and maintained at 4 °C. NaBHCN, iodoacetamide (IAM), dithiothreitol (DTT), guanidine hydrochloride (GuHCl), ammonium bicarbonate (NH₄HCO₃), and 2-AB- labeling Kit were purchased from Sigma. Trypsin, peptide N-glycosidase (PNGase F) were obtained from Shanghai Zhangjiang Biotech, Inc. Formic acid (FA) was obtained from EM sciences, Optima-grade acetonitrile (ACN) was obtained from Fisher Scientific, and HILIC solid phase extraction (SPE) posts were obtained from Agela Corp.

2.2. Methods

2.2.1. Intact protein analysis

rHuPH20 (50 µg) was digested with PNGase F (5 µg) (1:10 w/w). The intact and digested samples were analyzed by reverse-phase LC-MS. The analysis of intact and digested samples was performed on a Waters MassPREP™ Micro Desalting Column (2.1 × 50 mm) using a gradient (6–7 min, 10–90% B). The mobile phase B was acetonitrile with 0.1% formic acid, while the mobile phase A was water with 0.1% formic acid. The flow rate was maintained at 0.40 mL/min, and the column temperature was maintained at 80 °C. Mass spectrometric analysis was performed on a Waters Xevo G2-S Q-TOF MS system with a positive ion mode. The desolvation gas and source temperature were set to 400 °C and 120 °C. The capillary and cone voltages were set at 3,000 and 35 V, respectively. Transfer collision energies were set at 0 V. The *m/z* scan range was set to 500–3500. The deconvolution of ESI mass spectra was performed by Biopharmalynx 1.3.3 using MaxEnt 1 algorithm.

2.2.2. Peptide mapping analysis

rHuPH20 samples were denatured with 8 M guanidine hydrochloride and further reduced with 15 mM DTT at 37 °C for 1 h. After cooling the samples at room temperature, the rHuPH20 samples were alkylated with 50 mM IAM in the dark for 45 min at room temperature. The reaction was stopped by adding 1 M DTT to the final concentration of 35 mM. Then samples were exchanged the buffer to 50 mM NH₄HCO₃ solution (pH 8.2) prior to trypsin digestion. The rHuPH20 samples were digested with trypsin (1:50 w/w) at 37 °C for 18 h, and the tryptic digests were terminated at 0.5% FA. The final tryptic digests were centrifuged at 17,000 g for 10 min, and the supernatant was transferred to a clean EP tube for LC-MS/MS analysis. The peptide mixture was separated on a BEH300C18 column (2.1 × 100 mm, 1.7 µm) (Waters, Milford, MA) with a column temperature of 45 °C using an 80-min linear gradient (1–37% B), and the total run time was 100 min. The column was equilibrated with 1% B in water. The LC mobile phases were the same as intact samples mass analysis above and the flow rate was maintained at 0.20 mL/min. Data was obtained with positive ionization. The eluted peptides were detected by MS with a changing low collision energy (6 V) and high collision energy (ramping from 25 to 45 V) ESI acquisition mode to obtain the precursor ions (MS) and their fragmentation data (MS^E), respectively. The ion source setup was presented as follows: capillary voltage, 3,000 V; the desolvation temperature, 350 °C; source temperature, 120 °C; cone voltage, 40 V; cone gas flow, 100 L/h; and the desolvation gas flow, 800 L/h; the *m/z* scan range was set to 50–1950, respectively.

The system was controlled by MassLynx 4.1 (Waters, Milford, MA),

and the LC-MS/MS data analysis was performed by BiopharmaLynx 1.3.3 software, using fully tryptic cleavage rules for the sequences of the protein. The mass accuracy was set to no more than 30 ppm for both precursors and fragments. The identified peptides were confirmed by MS^E spectra with at least three fragment ions.

2.2.3. Free glycan profiling

N-linked glycans were released by PNGase F (1:10 w/w) digestion at 37 °C overnight, under denaturation conditions. After precipitating deglycosylated samples with ice-cold ethanol for 30 min and centrifuging the mixture at 14,000 g for 30 min, the supernatant was transferred to a new sample tube and evaporated to dryness. Released and dried oligosaccharides were labeled with 2-aminonbensamide (2-AB) solution for 4 h at 65 °C. HILIC SPE post was used to remove excess labeling agents again. The flow-through containing the labeled oligosaccharides was transferred to a new sample tube and evaporated to dryness. The labeled N-glycan was reconstituted with 70% ACN in water prior to analysis.

Subsequently, the labeled glycans were separated on a hydrophilic interaction chromatography (HILIC) column (2.1 × 150 mm, glycan, 1.7 μm) at 60 °C and detected by an ACQUITY UPLC fluorescence detector plus Q-TOF MS. Separations were performed at a 45 min gradient (72–62% B) and 0.4 mL/min flow rate using 30 mM ammonium formate, pH 4.5 as the mobile phase A and 100% ACN as the mobile phase B. The desolvation gas and source temperature were set to 350 °C and 120 °C. The capillary and cone voltages were set at 3,000 and 45 V, respectively. Transfer collision energies were set at 6 V. The *m/z* scan range was set to 500–3000.

2.2.4. In vitro and in vivo assays for bioactivity of rHuPH20

The activity of hyaluronidase was measured using a Microturbidity Assay *in vitro*. Dilutions of the known PH20 were prepared in an enzyme diluent solution to generate a standard curve and 30 μl of each was added to a 96-well plate in triplicate. The enzyme diluent solution was prepared by dissolving of hydrolyzed galatin in 25 mL of the 50 mM PIPES reaction buffer (50 mM PIPES, 140 mM NaCl, PH 5.5) and 25 mL water, and adding 0.25 mL 20% human serum albumin solution into the mixture and vortexing for 30 s. The dilutions included 0 U/mL, 0.78 U/mL, 1.56 U/mL, 3.12 U/mL, 6.25 U/mL, 12.5 U/mL, 25.0 U/mL, 50 U/mL and 100 U/mL. Reagent blank wells were added to 60 μl enzyme diluent solution as a negative control. Then the plate was shaken for 10 s and warmed at 37 °C for 5 min. After shaking, a 30 μl substrate solution (prepared by 9 mL water, 10 mL PIPES and 1 mL 5 mg/mL sodium hyaluronate) was added to each well. The sodium hyaluronate was prepared by dissolving 100 mg of sodium hyaluronate in 20 mL water and mixed by gently rotating at 4 °C for 2–4 h. The plate was shaken for 10 s and warmed at 37 °C for 10 min.

The reaction was stopped by adding 240 μl serum working solution [25 mL serum stock solution (1 volume of human serum was diluted with 9 vol of 500 mM acetate buffer solution, pH 4.3, and the pH was adjusted to 3.1 with HCl) in 75 mL of 500 mM acetate buffer solution, pH 4.3] to each well. The plate was shaken for 10 s. After 15 min, the turbidity was measured at 640 nm and hyaluronidase activity was determined by fitting to the standard curve.

The activity of hyaluronidase was measured using interstitial dispersion experiments *in vivo*. All protocols were approved by the animal care. Nude mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg) followed by injection of sample or carrier control (10 mM Hepes, 1 mM Calcium, 130 mM NaCl, 1 mg/mL Human Serum Albumin) in pressure perfusion apparatus in a final volume of 50 μl. The sample and control were isotonic and their pH values ranged from 6.5 to 7.5. rHuPH20, hyaluronidase reference standard or carrier control were injected with 50 μl of trypan blue dye at 0.5, 1, 24, and 48 h. Following injection, dye areas were measured at the respective time points, under normal light (trypan blue) using microcalipers and measured in 2 dimensions, with areas calculated using the formula $\text{Area} = ((D1 \times D2) \times \pi/4)$ (where D1 and D2 are the diameters of the dye

front) [19].

2.2.5. Pharmacokinetics and bioavailability of atezolizumab

Anesthetized Sprague–Dawley rats (6 per group) were injected intravenously (group 1), subcutaneous (group 2), subcutaneous with 100 Units rHuPH20 (group 3) and subcutaneous with 100 Units rHuPH20 treated with PNGase F (group 4) in a 150 μL volume with atezolizumab (50 mg/kg), respectively. About 300 μL of blood was collected via tail bleed at 0.5, 1, 2, 4, 8, 24, 48, 72, 96 and 120 h post-dose from animals receiving atezolizumab. For pharmacokinetic assessments, atezolizumab concentration of was measured in serum by ELISA. Pharmacokinetic parameters were calculated using the linear trapezoidal rule with integration from T₀ to infinity based upon the elimination rate constants established from the intravenous doses.

3. Results and discussion

3.1. Analysis of the intact rHuPH20 deglycosylated with PNGase F

Mammal animal hyaluronidase containing extracts from testes have been used for over 50 years to increase tissue permeability and facilitate the dispersion and absorption of other co-injected drugs. Despite their relative safety and potency, they have generally been limited by both immunogenicity and impurity profiles. The confounding activities of contaminants present in crude hyaluronidase preparations, such as proteinases, anti-coagulants, growth factors and other unidentified components may in fact result in the various biological properties, such as being pro-inflammatory or proangiogenic [19]. To overcome the disadvantages of mammal animal hyaluronidase derived extracts, we generated the rHuPH20 in CHO cells. The recombinant enzyme is a soluble single chain polypeptide with six potential N-linked glycosylation structures lacking the GPI-anchor that, like the bovine PH20, degrades hyaluronan by hydrolyzing the β 1,4 linkage between the C1 position of N-acetyl glucosamine and the C4 position of glucuronic acid.

Therefore, this characterization is necessary for rHuPH20 because the non-human glycoforms may potentially be immunogenic and they must be identified, quantified, and may be limited when the rHuPH20 is intended for therapeutic use.

A combination of UPLC and ESI-Q-TOF MS was used to measure the molecular masses at the intact protein level. The rHuPH20 was digested with PNGase F to remove the N-linked glycans. Data processing software (BiopharmaLynx 1.3.3) was used to automatically deconvolute the collected data to a single peak, which represents the average mass for each liquid chromatography peak. The deconvoluted mass spectrum of the rHuPH20 digested with PNGase F was shown in Fig. 1. From this spectrum, two main peaks were identified. The main peak molecular weight was 66,465 Da, which corresponded to the intact rHuPH20. The sub-peak molecular weight was 52,261 Da, which corresponded to the rHuPH20 whose N-linked glycans were removed using PNGase F. rHuPH20 has 6 potential N-glycosylation sites, and after PNGase F treatment, the molecular weight was reduced by approximately 14 kDa; therefore, the result demonstrated that most, if not all, of the sites are linked to oligosaccharides. This is based on the assumption that the observed mass (52 kDa) of the deglycosylated sample was consistent with the theoretical molecular weight of amino acids sequence.

3.2. Analysis of total N-linked released glycans of the rHuPH20

rHuPH20 contains six potential N-glycosylation sites which are candidates for glycosylation: Asn47, Asn131, Asn200, Asn219, Asn333, Asn358. The released N-linked glycans from rHuPH20 treated with PNGase F were labeled with 2-AB, separated using a HILIC column, and quantified by UPLC with a fluorescence detector. The complete release of N-glycans from rHuPH20 was further confirmed by SDS-PAGE, the result of SDS-PAGE analysis indicated that there was nearly 14 kDa less in apparent molecular mass after PNGase F digestion (52KD) than before

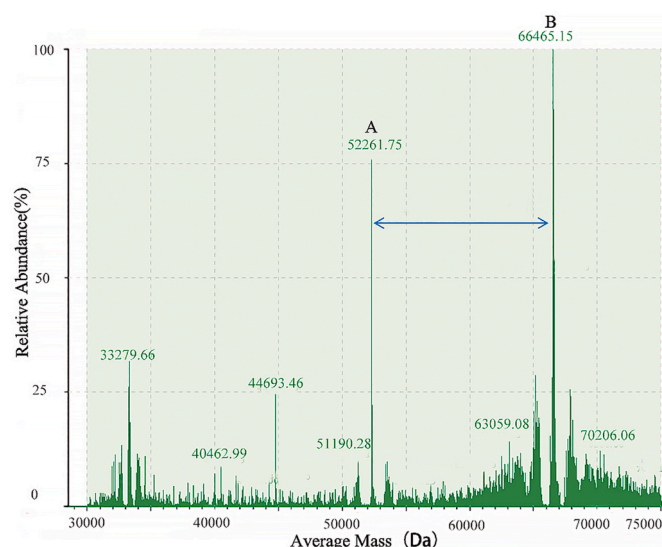


Fig. 1. The deconvoluted mass spectrum of rHuPH20 after PNGase F treatment. B and A are the mass of glycosylated and deglycosylated rHuPH20, respectively.

(66KD) (Fig. S1). The fluorescence chromatograms of 2-AB labeled glycans separated by the UPLC HILIC column was shown in Fig. 2. Using the ESI-Q-TOF mass spectra, the glycan mass profiling and structure elucidation were performed. Ten major glycans (G0F, Man5, G1F, Man6, G2, G2F, Man7, G2F + Nac, G3F, G4F; Fig. 2 and Table 1) were

identified and quantified based on the integrated peak area. The result was consistent with the data of the intact rHuPH20 digested with PNGase F by LC-MS.

A typical CHO-derived recombinant IgG has abundant G0F, low G1F/G2F, trace amounts of sialic acids with some materials having some high-mannose (Man5–Man9) structures [28,29]. Levels of high mannose glycans on recombinant antibodies can range from 1% to greater than 20% [30]. However, according to the integrated peak area, glycan profiles differed between subtypes, with greater levels of more fully galactosylated species (e.g. G2F, G3F, G4F) and a small amount of G1F and G0F, whereas, the rHuPH20 had a much higher high-mannose-type glycans than recombinant IgGs.

3.3. Site-specific N-glycosylation analysis of the rHuPH20 by tryptic peptide mapping

While MS analysis of intact protein is a useful and fast assay, it cannot exactly identify the glycosylation site. MS/MS methods based on analysis of intact glycopeptides provide more information on the spectra of glycans at individual glycosites. To further explore the details of modifications and variants, LC-MS^E peptide mapping was performed. In general, confident characterization of the six potential intact glycopeptides is a challenging task due to microheterogeneity of glycosylation, complexity of glycans, and insufficient fragmentation of peptide bones. Exceptional resolution is possible because separations obtained through the use of UPLC's small particles result in dramatically narrower glycopeptide peaks.

Peptide mapping was a useful technique for certifying the proteins'

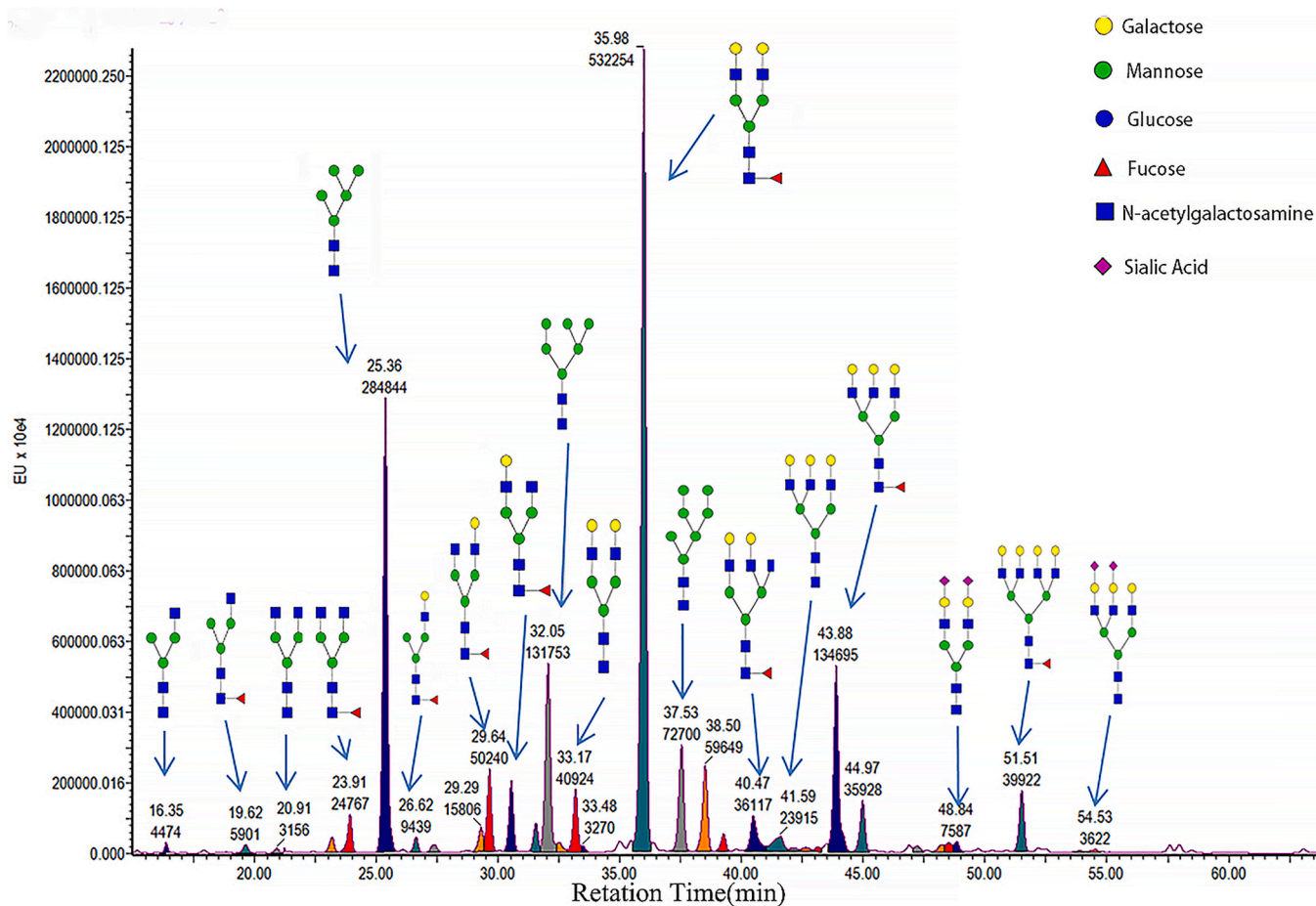


Fig. 2. Free glycan profile of rHuPH20 detected by the fluorescence detector and confirmed by MS. The N-glycans were removed from rHuPH20 with PNGase F. The released glycans was labeled with 2-AB, and analyzed by HILIC-FLR-MS.

Table 1
N-Glycans released from the rhuPH20 with PNGase F.

RT(min)	Glycan	Composition	Proposed structure	Theoreticalmass (da)	Observed mass (da)	Δ mass (ppm)	FLR Area%
16.35	G0-Nac	Hex3HexNAc3		1234.4832	1234.5493	53.5	0.27
19.62	G0F-Nac	Hex3HexNAc3DHex1		1380.5411	1380.5394	-1.23	0.36
20.91	G0	Hex3HexNAc4		1437.5625	1437.5570	-3.82	0.19
23.91	G0F	Hex3HexNAc4 DHex1		1583.6205	1583.6208	0.189	1.49
25.36	Man5	Hex5HexNAc2		1355.5095	1355.5082	-0.96	17.16
26.62	G1F- Nac	Hex4HexNAc3 DHex1		1542.5939	1542.5909	-1.94	0.57
29.64	(1,6)G1F	Hex4HexNAc4 DHex1		1745.6733	1745.6674	-3.38	3.03
30.54	(1,3)G1F	Hex4HexNAc4 DHex1		1745.6733	1745.6681	-2.98	2.65
32.05	Man6	Hex6HexNAc2		1517.5623	1517.5583	-2.64	9.07
33.17	G2	Hex5HexNAc4		1761.6682	1761.667	-6.81	2.47
35.98	G2F	Hex5HexNAc4 DHex1		1907.7261	1907.7236	-1.31	32.39
37.53	Man7	Hex7HexNAc2		1679.6151	1679.6119	-1.91	7.97
40.47	G2F + Nac	Hex5HexNAc5 DHex1		2110.8055	2110.8176	5.73	2.93
41.59	G3	Hex6HexNAc5		2126.8004	2126.7964	-1.88	1.14
43.88	G3F	Hex6HexNAc5 DHex1		2272.8583	2272.8442	-6.20	10.5
48.84	G2+2SA	Hex5HexNAc4NeuAc2		2343.8590	2343.8579	-0.47	1.38
51.51	G4F	Hex7HexNAc6dHex1		2637.9905	2637.9863	-1.59	2.71
54.53	G3+2SA	Hex6HexNAc5NeuAc2		2708.9912	2708.9802	-4.06	0.22

amino acid sequences and their PTMs, including glycosylation, oxidation and deamidation [31]. The peptide mapping method could also be used as an alternative or be combined with an oligosaccharide mapping method to acquire site-specific information for proteins with multiple glycosylation sites.

Consequently, peptide mapping was used to further study rHuPH20 glycosylation. Using a data acquisition C18 LC-MS with alternate chargeable energy full scanning (LC-MS^E) was useful for glycopeptide characterization [32]. Compared with LC-UV/MS peptide mapping methods, MS^E acquired exclusive fragments of all precursors. Consequently, all peptide precursors were fragmented in one experiment and got all the information with full-scan fragments. Chakraborty and colleagues had applied LC-MS^E for analyzing peptide maps of a protein digest [33]. High sequence coverage and good analytical reproducibility were reached in replicated analyses of peptide.

To determine the oligosaccharide heterogeneity at each glycosylation site, reduced and carboxymethylated rHuPH20 was digested into peptides and glycopeptides. A mixture of tryptic peptides and glycopeptides was subjected to LC/ESI/MS/MS with a reversed-phase column. Fig. 3A shows a total ion chromatogram (TIC) of the TOF MS scan for the full scan *m/z* 50–1950. Fig. 3B shows a TIC of the product ion scan. Because product ion spectra of glycopeptide precursor ions have abundant carbohydrate b-ions, *m/z* 204 (HexNAc), *m/z* 186 (HexNAc-H₂O), *m/z* 366 (HexHexNAc), and *m/z* 292 (NeuAc), the extracted ion chromatogram at *m/z* 204 and *m/z* 366 of the product ion scan are illustrated in Fig. 3C and D, respectively. The extracted ion chromatogram at *m/z* 204 and *m/z* 366 of product ion spectra provides a useful

information of the selection of glycopeptide precursor ions. The glycopeptide ions were assigned based on an examination of product ion spectra using the information on amino acid sequences of the six peptides containing a putative N-glycosylation site [34].

Consistent with the our expectations, all the putative sites for N-glycosylation were found to carry oligosaccharide units. All tryptic peptides contained the N-glycosylation consensus sequence N-X-S/T (T10-12: NWKPKDVYKNR at 17.51min, T4: INATGGQVTIFYVDR at 43.34 min, T19-21: LLRPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIR at 47.92 min, T34-35: KWNSSDYHLNLPDNFAIQLEK at 51.23min, T21-22: RNDLWSLWNESTALYPSIYLNTQQSPVAATLYVR at 68.66 min, T31-32: SMKSCLLDNYMETILNPYIINVTLAAK at 74.05 min, Fig. S2). As expected, the major glycoforms were fully galactosylated species in T10-12(Fig. 4A), T4 (Fig. 4B), and T21-22 (Fig. 4E), which consistent with those of the proteins expressed in CHO cells. Compared to T4, T10-12 and T21-22, high-mannose-type glycans were the major glycoforms in T19-21(Fig. 4C), T34-35(Fig. 4D), and T31-32(Fig. 4F). This glycosylation pattern may play an important role in the biological activity of rHuPH20. As expected, G1F and G2F are commonly observed on the Fc region of antibodies produced in CHO cells. High-mannose-type N-glycans are commonly found in glycoproteins derived from yeasts, insect cells, and plants. Although the majority of therapeutic proteins contain very low levels of high-mannose structure, a high levels of high-mannose-type N-glycans (Man5 and Man6) are also found in rHuPH20 produced in CHO cells. High-mannose-type N-glycans may result in a high immunogenicity and decreased serum half-life in humans. Therefore, care may be taken to control the mannose content in

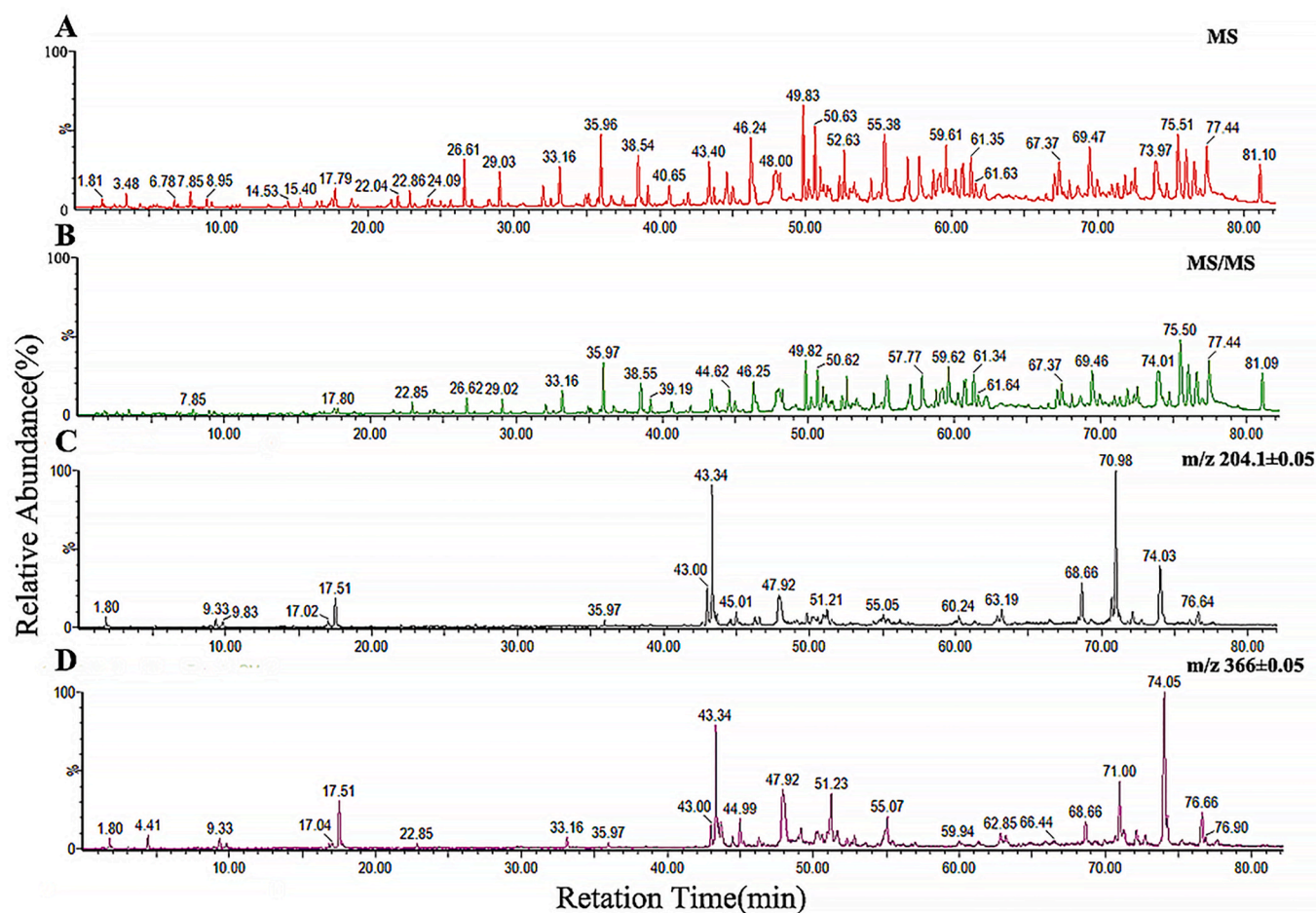


Fig. 3. The tryptic peptide mapping of rHuPH20. A is a total ion chromatogram (TIC) of a TOF MS scan for the full scan m/z 50–1950. B is a TIC of the product ion scan. C and D are the extracted ion chromatogram at m/z 204 (HexNAc) and m/z 366 (HexHexNAc) of the product ion scan.

therapeutic proteins.

While peptide mapping revealed the glycosylation sites of rHuPH20, the released and labeled glycans analyzed by HILIC with fluorescence detection was used for accurate quantification of the glycans. As shown in Fig. 2, G2F and Man5 were the major glycoforms of rHuPH20. The result was consistent with the assay values determined by peptide mapping method.

3.4. *In vitro* and *in vivo* bioactivity analysis of the rHuPH20

To determine the influence of N-linked glycans on rHuPH20, the activity of rHuPH20 was measured using turbidity assay *in vitro* and dye diffusion assay *in vivo*. Hyaluronidase activity of rHuPH20 *in vitro* was determined using a turbidometric assay, which is based on the formation of an insoluble precipitate when hyaluronic acid binds with serum albumin [35]. The activity is measured by incubating rHuPH20 with soluble hyaluronate (hyaluronic acid). The turbidity of the resulting sample is measured at 640 nm after a 30 min development period. The decrease in turbidity resulting from enzyme activity on the sodium hyaluronate substrate is a measure of rHuPH20 activity. The method is performed using a calibration curve generated with dilution of a soluble rHuPH20 assay working reference standard, and using it to automatically back calculating unknown sample activity.

A calibration curve was constructed, and the activity of rHuPH20 is 120000 U/mg. However, the activity of the rHuPH20 after PNGase F treatment decreased by approximately 80% (Fig. 5A). This result demonstrated that N-glycosylation of the rHuPH20 is necessary for its hyaluronidase activity.

To examine the bioactivity of rHuPH20 *in vivo*, 5 Units of purified rHuPH20, 5 Units of digested rHuPH20 (PNGase F) and 5 Units of a bovine tested hyaluronidase preparation or carrier control were injected by subcutaneous injection in nude mice. 50 μ L trypan blue dye, a 960 Da molecular weight tracer, was added to the enzyme or carrier to visualize the extent of diffusion. As shown in Fig. 5B, the dispersion area of trypan blue dye was almost equal between the 5 Units of purified rHuPH20 and 5 Units of a bovine tested hyaluronidase preparation. The dispersion area of trypan blue dye was significantly increased relative to carrier controls at 20 min post-injection using either enzyme. However, the dispersion area of trypan blue dye of digested rHuPH20 (PNGase F) was significantly decreased (Fig. 5B). This result demonstrated that N-glycosylation of the rHuPH20 is also necessary for its bioactivity, and the result was consistent with *in vitro* data. As shown in Fig. 5C, by 24 h post-hyaluronidase injection, the tracer dye area was indistinguishable from that generated at the carrier control or digested rHuPH20 (PNGase F) injection sites, demonstrating that the changes induced by rHuPH20 in the interstitial matrix were reversible and occurred within a time frame similar to the reported half-life of hyaluronan in the skin [19].

3.5. Pharmacokinetic profiles and bioavailability of atezolizumab with rHuPH20

Many drugs must be injected intravenously due to limitations on drug solubility, bioavailability or required drug volume, especially for therapeutic antibodies. The speed of absorption may be reduced by the structure of subcutaneous tissues, which mainly include hyaluronan, a glycosaminoglycan of the extracellular matrix [36]. Pharmacokinetic

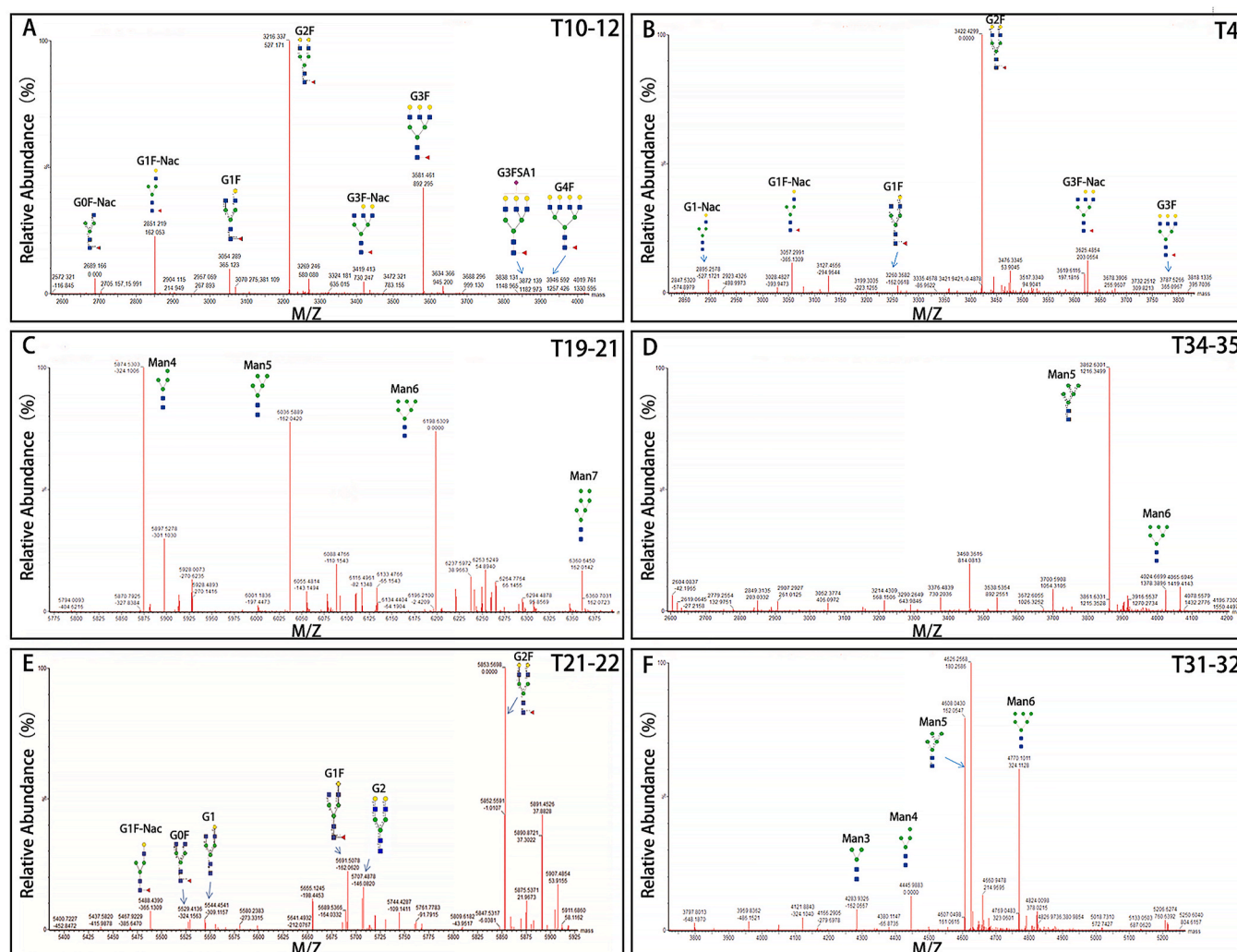


Fig. 4. MS/MS spectra of precursor ions for peptide T10-12(A), T4(B), T19-21(C), T34-35(D), T21-22(E) and T31-32(F).

profile could be altered by increasing the exposure of therapeutics to the capillary beds and lymphatics [19]. As therapeutic protein can be degraded by cells of the interstitial matrix, reducing the residence time in this compartment may increase systemic bioavailability of therapeutic protein. To prove the concept and evaluate the effect of N-glycosylation on rHuPH20, we assessed the effects of rHuPH20 on the bioavailability and pharmacokinetics of atezolizumab as the model biotherapeutics in a rodent model. Four groups of 5 rats each received atezolizumab at a concentration of 50 mg/kg administered either IV or locally SC with and without 100 U of rHuPH20 or 100 U of digested rHuPH20. As shown in Fig. 5D, the absolute bioavailability of the atezolizumab increased from 48.8% to 78.2% ($P < 0.05$). In addition, T_{max} decreased from 48 to 24 h ($P < 0.05$), indicating that rHuPH20 markedly improved the pharmacokinetic profiles of atezolizumab administered via the interstitial route. After PNGase F treatment of the rHuPH20, the absolute bioavailability of the atezolizumab decreased, and T_{max} also increased from 24 to 48 h ($P < 0.05$). Those results demonstrated that glycosylation of rHuPH20 play essential roles for its bioactivity. Those results were consistent with available *in vitro* and *in vivo* data. Co-administration with rHuPH20 permitted the administration of up to five times more volume than normally feasible for subcutaneously injected drugs. By increasing the infusion rates, co-administration with rHuPH20 reduced the tissue interstitial matrix for systemic adsorption, potentially enabling subcutaneous injections of intravenous dosage.

Our studies also demonstrated that the pharmacokinetic profiles of

antibody drugs injected subcutaneously could be significantly improved by co-administration with rHuPH20. We selected a therapeutic antibody atezolizumab from among the many therapeutic proteins that are presently marketed. Atezolizumab is a high-affinity engineered anti-PD-L1 monoclonal IgG1 antibody that inhibits the interaction of PD-L1 with PD-1 and B7.1 (CD80) [37]. The antibody is administered by intravenous infusion due to the large dose volume. Comparison between the areas under the curve of atezolizumab delivered intravascularly or subcutaneously showed that the pharmacokinetic profile and absolute bioavailability of atezolizumab were dramatically improved when co-administered with rHuPH20. As a result, atezolizumab co-administered with rHuPH20 exhibited comparable pharmacokinetic profiles to intravenous dosing. Therefore, this may allow many drugs which need intravenously-administered to be administered by subcutaneous injection, and may make it possible for patients with immunological diseases to self-administer injectable medicines, and improve patient compliance.

4. Conclusion

In this study, UPLC and tandem mass spectrometry was used to characterize the rHuPH20 expressed in CHO cells. Removal of the N-linked glycans from rHuPH20 with PNGase F shifted the molecular weight from 66 kd to approximately 52 kd, its deduced molecular weight based on sequence analysis, suggesting that most, if not all, of the

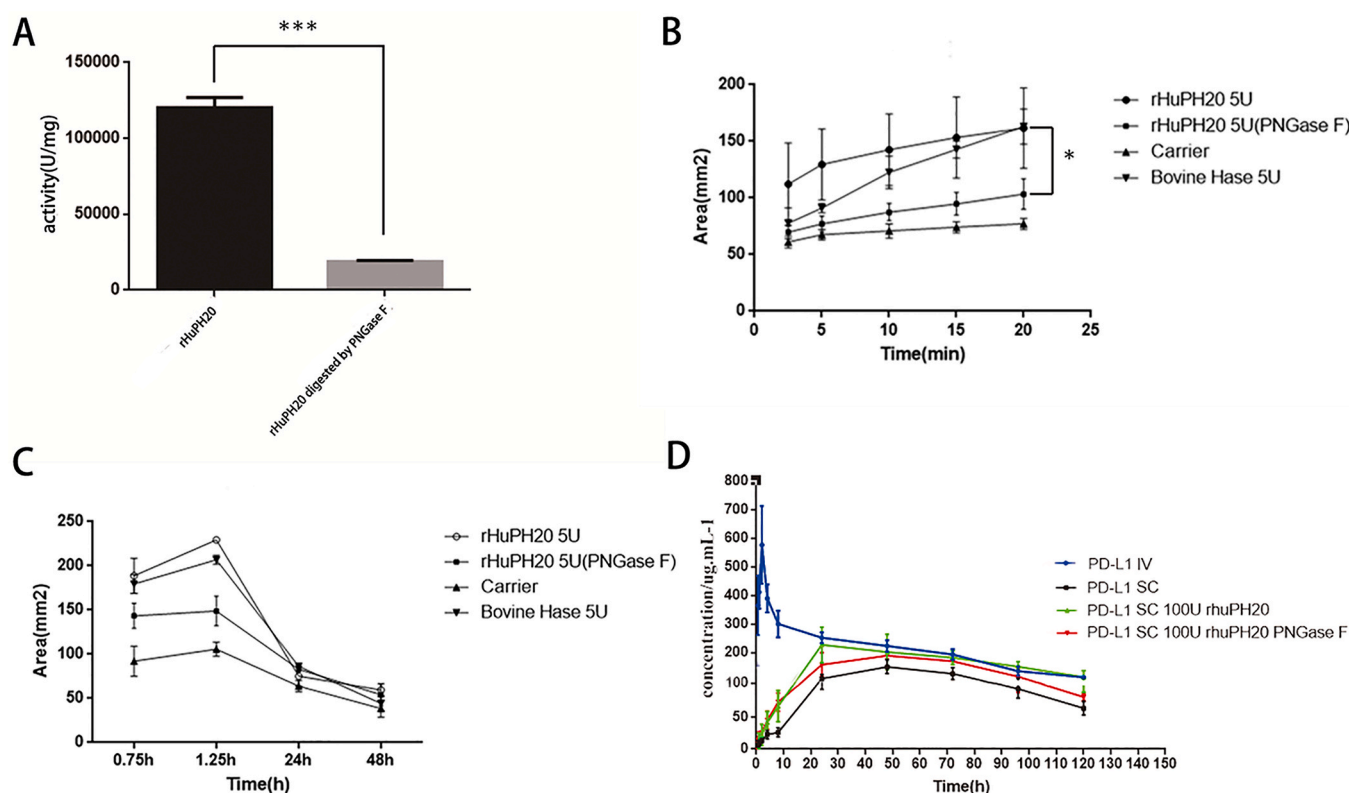


Fig. 5. Analysis of the bioactivity of the rHuPH20 *in vitro* and *in vivo*. The activity of rHuPH20 after PNGase F treatment decreased by approximately 80% (A). The dispersion area of trypan blue dye of 5 Units of digested rHuPH20 (PNGase F) was significantly decreased (B). The tracer dye area has little difference from that generated at the carrier control injection sites by 24 h post-hyaluronidase injection as shown in C. The pharmacokinetic profiles of atezolizumab (D). (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

potential N-glycosylation sites are linked to oligosaccharides. Then the N-glycans released by PNGase F were characterized using UPLC-FLR-MS. The galactosylated and high mannose glycans (Man5, G1F, Man6, G2F, Man7, G3F, G4F, etc.) were identified and quantified based on the integrated peak area. The six N-glycosylation sites were further identified and characterized by UPLC-MS/MS at peptide levels for the first time. Subsequently, we found that the rHuPH20 increased the dispersion of locally subcutaneous injected therapeutic antibody and the *in vitro* and *in vivo* bioactivity were decreased significantly after PNGase F treatment. In particular, rHuPH20 can significantly augment the absolute bioavailability of locally subcutaneous injected antibodies, while the bioavailability decreased after PNGase F treatment of the rHuPH20. This study proved that N-glycosylation of the rHuPH20 most, if not all, is necessary for its bioactivity.

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CRediT authorship contribution statement

Tao Liu: Data curation, Writing – original draft. **Yantao Li:** Methodology, Writing – original draft. **Jin Xu:** Software, Investigation, Formal analysis, Visualization. **Qingcheng Guo:** Investigation. **Dapeng Zhang:** Investigation. **Lankun Song:** Investigation. **Jun Li:** Investigation. **Weizhu Qian:** Validation. **Huaizu Guo:** Writing – review &

editing. **Xinli Zhou:** Supervision, Writing – review & editing. **Sheng Hou:** Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ab.2021.114380>.

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