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# Construction and analysis of a novel peptide tag containing an unnatural N-glycosylation site

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ABSTRACT

**The addition of N-glycans to clinically used proteins enhances their therapeutic features. Here we report the design of a novel peptide tag with an unnatural N-glycosylation site, which may increase the N-glycan content of generally any protein. The designed GlycoTags were attached to A1AT, EPO and AGP and constructs were expressed in HEK293 or CHO cells. Hereby we could prove that the attached unnatural N-glycosylation site is decorated with complex-type N-glycans and that the spacer as well as the C-terminal "tail" sequence are critical for the usage of the novel N-glycosylation site. This demonstrates that the novel GlycoTag is a convenient tool to provide proteins with extra N-glycan moieties by simply adding a peptide tag sequence as small as 22 amino acids.**


## 1. Introduction

In the last years, the importance of recombinantly expressed therapeutical (glyco)proteins – named biotherapeutics – has remarkably increased. These include cytokines like erythropoietin, hormones or numerous antibodies that have been developed for the treatment of cancer or inflammation [1]. It is well known that the carbohydrate moieties of biotherapeutics play a key role in influencing the stability as well as the biological function of glycoproteins [2]. In particular, the circulatory half-life of serum glycoproteins is directly depending on the amount of sialic acids that they contain; desialylated glycoproteins are cleared from the circulation by binding to the asialoglycoprotein receptor and/or mannose receptor [2–4].

The influence of glycan moieties on the activity and half-life of proteins was impressively illustrated by a modified erythropoietin (EPO) molecule. This genetically engineered Darbepoietin alfa carries two additional N-glycans besides three natural N-glycans and one O-glycan and exhibits a threefold longer half-life than the natural EPO [5]. Interestingly, Darbepoietin alfa has a fourfold lower receptor binding activity. However, the activity is sufficient to activate the EPO receptor, so that the overall potency in increasing the hematocrit is about 13-fold more potent than the natural EPO when administered once weekly [5]. Thus, the glycan moieties of recombinant proteins are ideal targets for the design of improved biotherapeutics.

The introduction of additional N-glycosylation sites into the coding sequence of a protein may lead to its inactivity because the expressed N-glycans or the mutated amino acids may change the steric properties or the three-dimensional structure of the protein. Therefore the introduction of a peptide tag constituted of an N-glycosylation site may overcome these disadvantages as the mature amino acid sequence of the protein is not changed. Furthermore the GlycoTag technology reported here presents the advantage that it can potentially be applied to any protein unless the N- or C-terminus is hidden inside the protein structure.

However, the utilization of an N-glycosylation motif at the C-terminus of a protein by the oligosaccharyl transferase complex (OST) depends on the structural features of the C-terminal amino acid sequence. A statistical study based on a data set of occupied and non-occupied N-glycosylation sites showed that non-occupied sites are found more frequently towards the C-terminal end [6]. This was later supported by a study in which the N-glycosylation efficiency was reduced if the C-terminus consisted of less than 68 amino acids [7]. Furthermore, an in vitro transcription/translation study of the *Escherichia coli* inner membrane leader peptidase showed that the N-glycosylation efficiency is reduced if the N-glycosylation site is placed less than approximately 60 residues

*Abbreviations:* A1AT, alpha-1 antitrypsin; AGP, alpha-1 acid glycoprotein; EPO, erythropoietin; OST, oligosaccharyl transferase; SP, signal peptide; GT, GlycoTag; CDS, coding sequence

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from the C terminus [8]. This correlation was also demonstrated for the prion protein [9] as well as for tissue plasminogen activator [10]. Besides this co-translationally controlled N-glycosylation, it is known that N-glycosylation can also occur post-translationally within 60 amino acid residues from the C-terminus. This is dependent on structural features of the glycosylated protein as it was shown that the N-glycosylation sites of membrane dipeptidase 26 residues [11] and a mutant form of peptidylglycine α-amidating monooxygenase 13 residues near the C-terminus [12] are efficiently utilised.

## 2. Materials and methods

### 2.1. Materials

Monoclonal anti-human A1AT HRP-conjugated antibody from sheep (The binding site, Schwetzingen, Germany); polyclonal rabbit anti-human A1AT (Dako A/S, Glostrup, Denmark); monoclonal anti-human EPO antibody from mouse (R&D Systems, Minneapolis, USA); monoclonal anti-human AGP antibody from mouse (Sigma, Missouri, USA); secondary antibody peroxidase-conjugated AffiniPure Rat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, Suffolk, UK); Zeocin (Invitrogen; Karlsruhe, Germany); Trypsin from bovine pancreas TPCK treated and N-Benzoyl-DL-arginine-4-nitroanilide-hydrochloride (BAPNA) (Sigma–Aldrich, Steinheim, Germany); AEM serum free medium, Zeocin and Lipofectamine (Invitrogen, Karlsruhe, Germany). Other materials were purchased from Sigma–Aldrich, Steinheim, Germany.

### 2.2. Construction of A1AT, EPO and AGP-GlycoTag variants

The human A1AT-cDNA was cloned from PslLoxAAT (kindly provided by V. Sandig, ProBioGen AG, Berlin, Germany), the human Epo-cDNA from pKex-cmvEPO (kindly provided by K. Bork, Universitätsklinikum Halle (Saale)) and the human AGP-cDNA from hAGP-pcDNA6V5HisA (kindly provided by S. Riese, Charité – Universitätsmedizin Berlin) into pcDNA3.1 zeo (+) vector from Invitrogen. A1AT-GT1.1 was amplified by PCR using the primers A1AT-N-EcoRI-fw GCG GAA TTC ATG CCG TCT TCT GTC TCG TG and A1AT-GT1.1-XbaI-rev C GCT CTA GAC GGT AGC GTT CTC GAG TTT TTG GGT GGG ATT CAC CAC. The GlycoTags GT1.2, GT1.3 and GT1.4 were introduced by ligation of synthetic dimeric oligonucleotides using the former introduced XhoI and XbaI sites. GT1.2-XhoI-XbaI-fw TC GAG GCT GCT AAC GCT ACC GT/GT1.2-XhoI-XbaI-rev CTA GAC GGT AGC GTT AGC AGC C; GT1.3-XhoI-XbaI-fw TC GAG GCT GCT GCT GCT AAC GCT ACC GT/GT1.3-XhoI-XbaI-rev CTA GAC GGT AGC GTT AGC AGC AGC AGC C; GT1.4-XhoI-XbaI-fw TC GAG GCT GCT GCT GCT GCT GCT AAC GCT ACC GT/GT1.4-XhoI-XbaI-rev CTA GAC GGT AGC GTT AGC AGC AGC AGC AGC AGC C; GT1.4tail-1-fw TC GAG GCT GCT GCT GCT GCT GCT AAC GCT ACC GTC GCG GCC GCT TCT GGT GAC GTT TGG GAC AT /GT1.4tail-1-rev CTA GAT GTC CCA AAC GTC ACC AGA AGC GGC CGC GAC GGT AGC GTT AGC AGC AGC AGC AGC AGC C.

### 2.3. Transfection of HEK293 and CHO cells and selection

HEK293 cells were cultured at 5% $CO_2$ atmosphere and 37 °C in DMEM with glutamine supplemented with penicillin/streptomycin and 10% FCS and CHO-K1 cells were cultured at 5% $CO_2$ atmosphere and 37 °C in HAMS-F12 with glutamine supplemented with penicillin/streptomycin and 10% FCS. Transfection was performed with Lipofectamine according to the manufacture's instructions and cells were selected in the presence of 100 μg/ml Zeocin.

### 2.4. Expression and purification of A1ATwt, EPOwt, AGPwt and GlycoTag proteins

Stably transfected HEK293 or CHO-K1 cells were grown to 50% confluency washed two times with PBS and cultivated for 24 h in AEM or OptiCHO medium, respectively. Subsequently the medium was removed and the cells resuspended in fresh AEM or OptiCHO medium and grown in shaking flasks for ten days. The cell cultures were centrifuged; the supernatant filtrated by a 0.22 μm filter and diluted 1:2 in water. The A1AT GlycoTag proteins were then purified by anion-exchange chromatography (MonoQ 5/50 GL, buffer A 0.5 × PBS, buffer B 0.5 × PBS + 1 M NaCl) followed by size-exclusion chromatography (Superdex 200 10/300 GL, running buffer 0.5 × PBS).

### 2.5. SDS-Polyacrylamide gel electrophoresis, electroblotting and immunostaining

Samples were prepared according to Laemmli [13] and proteins were either directly visualized by Coomassie staining or transferred to a nitrocellulose membrane. Electrotransfer of proteins was performed in a tank trans-blot cell (Bio-Rad, Richmond, CA, USA) with blotting buffer (25 mM Tris, 114 mM glycine, 10% (v/v) ethanol). After blotting, the membrane was blocked with PBS containing 5% dry skim milk. A1AT was detected by anti-A1AT antibody conjugated with HRP, EPO was detected with a mouse anti-EPO antibody and AGP was detected with a mouse anti-AGP antibody, following incubation with the secondary anti-mouse antibody and detection carried out by the VersaDoc system (Bio-Rad, Richmond, CA, USA). Chemiluminescence signals were quantified using Quantity One software version 4.6.3.

### 2.6. A1AT-ELISA

The assay was performed in a 96-well micro plate. Each well was coated with 100 μl of polyclonal rabbit anti-human A1AT antibody (0.14 μg/ml in PBS) for 16 h at 4 °C. All following incubation steps were carried out at room temperature. The wells were subsequently blocked with 200 μl of 1% (w/v) BSA and 0.2% (v/v) Tween 20 in PBS for 2 h. 100 μl of standard (purified recombinant human A1ATwt) and samples were tested in triplicate and incubated for 2 h. The plate was finally washed with PBS containing 0.2% (v/v) Tween 20. Bound A1AT was detected with 100 μl monoclonal sheep anti-human A1AT HRP conjugated antibody.

### 2.7. A1AT-activity assay

The enzymatic activity of A1AT was indirectly measured by its inhibitory activity against trypsin, while the activity of trypsin was measured by its substrate N-Benzoyl-DL-arginine-4-nitroanilide-hydrochloride (BAPNA). Before measurement all samples were adjusted to a concentration of 60 μg/ml using the A1AT-ELISA and placed in a 96-well micro plate. A1AT (0.3 μg) was preincubated with trypsin (0.5 μg) in a total volume of 10 μl PBS at 37 °C for 10 min. Subsequently, 90 μl of BAPNA solution (2.75 mM in 100 mM NaCl, 0.01% (v/v) Triton X-100, 15 mM Tris pH 7.4) was added. The plate was incubated at 37 °C for 40 min and the absorption was measured at 405 nm. Recombinant human A1ATwt was used as the standard.

### 2.8. N-glycan analysis

The A1AT GlycoTag proteins were analysed as total pools. Analysis of N-glycans of the various A1AT-GlycoTag constructs were performed as previously described [14]. Briefly, N-glycans of purified A1ATwt and A1AT GlycoTag proteins were released by