Production and characterization of mono-PEGylated alpha-1 antitrypsin for augmentation therapy

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Abstract:

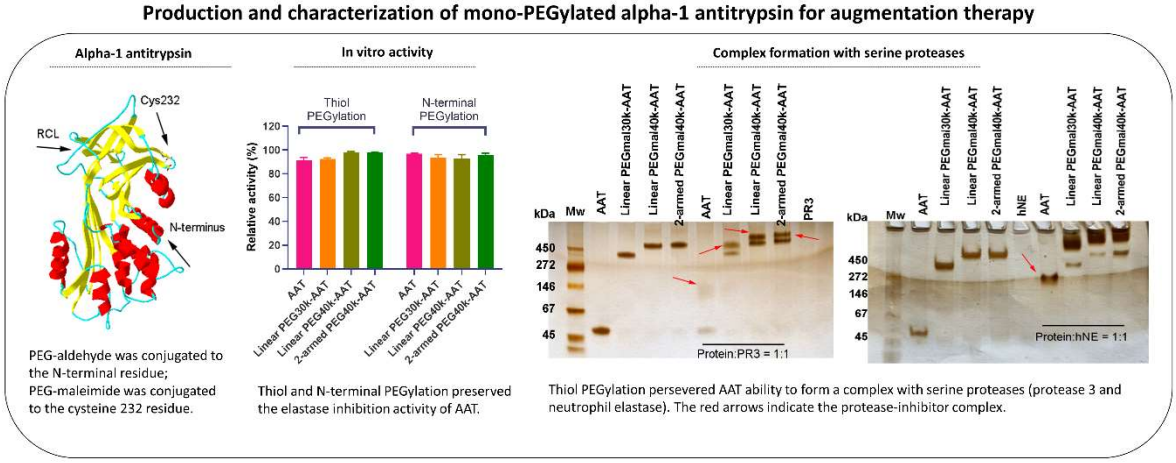
Alpha-1 antitrypsin (AAT) is an endogenous inhibitor of serine proteases which, in physiological conditions, neutralizes the excess of neutrophil elastase and other serine proteases in tissues and especially the lungs. Weekly intravenous infusion of plasma-purified human AAT is used to treat AAT deficiency-associated lung disease. However, only 2 % of the AAT dose reaches the lungs after intravenous infusion. Inhalation of AAT might offer an alternative route of administration. Yet, the rapid clearance of AAT from the respiratory tract results in high and frequent dosing by inhalation and limited efficacy. In the present study, we produced and characterized in vitro a PEGylated version of AAT which could offer a prolonged body residence time and thereby be useful for augmentation therapy by the intravenous and inhalation routes. Two PEGylation reactions – N-terminal and thiol PEGylation – and three polyethylene glycol (PEG) chains – linear 30kDa, linear 40kDa and 2-armed 40kDa – were used. The yields of mono-PEGylated AAT following purification by anion exchange chromatography were 40 – 50 % for N-terminal PEGylation and 60 – 70 % for thiol PEGylation. The PEG-AAT conjugates preserved the ability to form a protease-inhibitor complex with neutrophil elastase and proteinase 3 as well as the full inhibitory capacity to neutralize neutrophil elastase activity. These results open up interesting prospects for PEGylated AAT to achieve a prolonged half-life and an improved therapeutic efficacy in vivo.

Keywords: Alpha-1 antitrypsin, chronic obstructive pulmonary disease, PEGylation, neutrophil elastase

Abbreviations:

AAT, Alpha-1 antitrypsin; AEC, anion exchange chromatography; COPD, chronic obstructive pulmonary disease; DLS, dynamic light scattering; ESI-Q-TOF, electrospray-ionization quadrupole time-of-flight mass spectrometry; FBS, Foetal Bocine Serum; FPLC, fast protein liquid chromatography; GHA, growth hormone antagonist; hNE, human neutrophil elastase; MHS, murine alveolar macrophages; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; $NaCNBH_3$, sodium cyanoborohydride; PEG, polyethylene glycol; PEG-ald, PEG-aldehyde; PEGmal, PEG-maleimide; PR-3, proteinase 3; PVDF, polyvinylidene fluoride; rhAAT, recombinant non-glycosylated human alpha-1 antitrypsin; rhDNase, recombinant human deoxyribonulease I; SDS, sodium dodecyl sulfate; TCEP, tris(2-carboxyethyl) phosphine; VEGF, vascular endothelial growth factor.

Graphic Abstract



1 Introduction

Alpha-1 antitrypsin (AAT) is a 52-kDa serine protease inhibitor primarily produced in the liver. Alpha-1 antitrypsin provides essential protection to the lung tissue against the action of proteolytic enzymes such as neutrophil elastase, proteinase 3 (PR-3) and cathepsin G. Alpha-1 antitrypsin deficiency is characterized by low AAT serum levels (< 11 µM) (Chotirmall et al., 2015; Tonelli and Brantly, 2010). AAT deficiency is a hereditary condition caused by a mutation in the SERPINA1 gene(Chapman et al., 2018). The retention of misfolded and polymerized Z type AAT mutant within hepatocytes causes hepatic damages and cirrhosis(Blanco, 2017). The imbalance between proteases and protease inhibitors in the lungs may lead to premature emphysema and chronic obstructive pulmonary disease (COPD)(Brebner and Stockley, 2013).

Current approved therapies for patients with AAT deficiency and respiratory disease is intravenous augmentation therapy(Tonelli and Brantly, 2010). Although intravenous infusion of highly purified human AAT is safe, high doses of AAT are required (60 mg/kg weekly) due to the low fraction of the dose (2 %) reaching the lung epithelial lining fluid(Griese and Scheuch, 2016). Moreover, the benefits of augmentation therapy on pulmonary exacerbation or progression of emphysema have not been conclusively demonstrated in randomized controlled clinical trials. An additional drawback of augmentation therapy is its high annual costs with an average of 100,000 dollars per patient(Lomas et al., 2016). Almost a third of known qualified subjects with AAT deficiency do not receive the therapy due to financial constraints(Tonelli and Brantly, 2010).

Inhaled AAT has been developed as an alternative administration route for augmentation therapy in AAT deficiency (Brand et al., 2009; Franciosi et al., 2015). The potential advantages of inhaled over intravenous AAT include the noninvasive administration, high lung concentrations, lower doses, lower costs, and improved convenience for the patients. However, these advantages are counterbalanced by the large amount of inhaled AAT required (in total about 200 mg daily)(Brebner and Stockley, 2013; Franciosi et al., 2015). In a phase II/III clinical trial, patients with emphysema due to AAT deficiency received a twice-daily inhalation of 80 mg AAT (Stolk et al., 2019). After 50 weeks of treatment, inhaled AAT did not prolong the time to first exacerbation. However, the first exacerbation involved fewer symptoms. The severity of the exacerbation, including the dyspnea degree, the sputum volume and sputum purulence(Pauwels et al., 2004), lessened with AAT treatment, indicating AAT possible role in reducing the incidence or severity of dyspnea (Stolk et al., 2019). The limited efficacy of inhaled AAT might result from the rapid clearance of AAT from the lungs. Proteolytic breakdown within or outside the reactive center loop of AAT (Goldklang et al., 2019), oxidation of methionine 351 or 358 (Li et al., 2009) and phagocytes uptake might be the causes of the short residence time of AAT in the lungs. Therefore, to improve the therapeutic efficacy of inhaled AAT, it might be beneficial to prolong its residence time in the lungs.

PEGylation is a common process used to improve the therapeutic value of a protein by prolonging its serum half-life (Pasut and Veronese, 2012). Several PEGylated proteins have been approved by the FDA and EMA for clinical use by injection (Rondon et al., 2021). In a few preclinical studies, conjugation of PEG to proteins also showed the potential to improve their local residence time in the lungs after pulmonary delivery. Cantin et al. reported that the conjugation of recombinant non-glycosylated human AAT (rhAAT) to 20 kDa PEG resulted in the retention of the conjugate in the bronchoalveolar lavage after intranasal instillation in mice for at least 48 h whereas the unmodified rhAAT was cleared within 24 h (Cantin et al., 2002) . Similarly, Zhu et al conjugated a 20 kDa PEG to a more thermal stable recombinant AAT expressed in E. coli (Zhu et al., 2018). The PEGylated version showed the same activity as the unconjugated AAT. Koussoroplis et al. conjugated anti-IL17A F(ab')2 to a 2-armed 40 kDa PEG and demonstrated that the PEGylated antibody fragment presented

increased pulmonary residence time and improved anti-inflammatory effect (Koussoroplis et al., 2014). Guichard et al. showed that the conjugation of linear 30 kDa PEG or 2-armed 40 kDa PEG to recombinant human deoxyribonulease I (rhDNase) sustained its presence in murine lungs for 15 days and a single dose of PEGylated rhDNase provided similar therapeutic efficacy to reduce DNA content in the lungs of ***β***-ENaC mice, a model of the cystic fibrosis lung disease, as 5 consecutive daily doses of unconjugated rhDNase (Guichard et al., 2021).

Larger PEG (≥30 kDa) chains have been shown more effective to increase protein residence time in the lungs than smaller PEG chains (≤20 kDa) (Guichard et al., 2021). However, they have not been investigated for pulmonary AAT delivery yet. In addition, rhAAT is unstable *in vivo* (cleared within 4 h in the circulation) and PEG-rhAAT only showed similar serum half-life as plasma-purified glycosylated AAT (Zhu et al., 2018). Thus, it might be more promising to PEGylate glycosylated AAT rather than rhAAT and to use large PEG chains (≥30 kDa) to increase AAT stability and to sustain its body residence time.

In this study, PEGylated versions of glycosylated AAT were produced and characterized in vitro. Plasma-purified glycosylated AAT was mono-PEGylated selectively on i) its N-terminal residue by reductive alkylation at acidic pH, or ii) its cysteine 232 residue by thiol PEGylation (Figure 1). The size and composition of PEG used included linear 30 kDa PEG, linear 40 kDa PEG and 2-armed 40 kDa PEG. The mono-PEGylated products were purified and characterized for their PEGylation degree, size, protease neutralization capacity and formation of the inhibitor-protease complex.

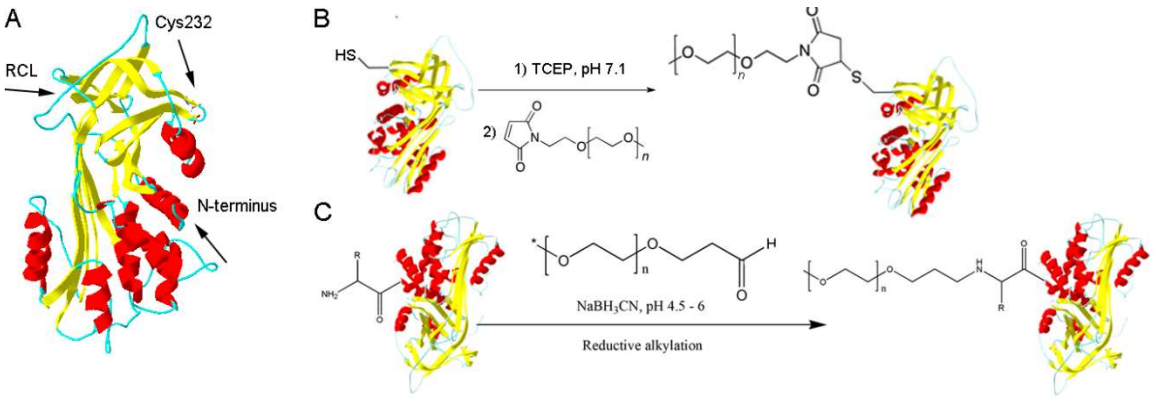


Figure 1. A) 3D structure of AAT (PDB: 2qug), generated by the SWISS-MODEL. The arrows show the reactive center loop (RCL) and the two PEGylation sites. B) Thiol PEGylation on the Cys232. C) N-terminal PEGylation

2 Materials and methods

2.1 Materials

AAT was purified from Pulmolast® (Lamepro, Breda, The Netherlands) by size exclusion chromatography (SEC; ÄKTA™ purifier 10 system, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using a HiLoad 16/600 Superdex 200 pg (GE Healthcare Europe GmbH, Diegem, Belgium) and 10 mM NaH_2PO_4 and 110 mM NaCl, pH 7.1, as eluent. This saline solution is the saline composition of Pulmolast® and it was also used for AAT storage at 4°C in our studies. The chromatograms were analyzed by UNICORN software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Through SEC purification, we were able to remove impurities which represented 23 ± 3 % of the Pulmolast® product (Supplementary Figure S1). Linear 30 kDa, 40 kDa and 2-armed 40 kDa methoxy-PEG-maleimide and linear 30 kDa, 40 kDa and 2-armed 40 kDa methoxy-PEG-aldehyde were purchased from NOF Europe GmbH (Frankfurt, Germany). Human neutrophil elastase (hNE) and its substrate N-Succinyl-Ala-Ala-Ala-p-nitroanilide were purchased from Elastin Products Company, Inc (Owensville, USA). All other reagents, unless otherwise mentioned, were obtained from Sigma-Aldrich, Inc (Overijse, Belgium).

2.2 Production of PEGylated AAT

AAT concentration was determined by the absorbance at 280 nm using Beer-Lambert Law with an extinction coefficient of 4.33 $(g/100\ mL)^{-1}\ cm^{-1}$ (A280, 1 %, 1 cm) (Chen et al., 2011). Prior to thiol PEGylation, AAT (5, 10 or 20 mg/ml) was reduced by tris(2-carboxyethyl) phosphine (TCEP) at a molar ratio of TCEP:AAT 4:1 in 10 mM NaH_2PO_4 and 110 mM NaCl, pH 7.1, at room temperature with mild agitation for 1 hour. PEG-maleimide was added to the reduced AAT at a PEG:protein molar ratio of 2:1 or 4:1. The TCEP was kept in the reaction mixture as it does not react with PEG-maleimide. The PEGylation was carried out under mild agitation for 1, 4 or 8 hours and stopped by adding 4-fold molar excess (comparing to the PEG reagent) of cysteine. The PEGylation yield was determined by anion exchange chromatography (AEC) at different time intervals.

For the N-terminal PEGylation, AAT (5 or 10 mg/ml) and 2, 4 or 8-fold molar excess of PEG-aldehyde were incubated in 20 mM succinate buffer, pH 6, in the presence of 120-fold molar excess (11.5 µM) of sodium cyanoborohydride ($NaCNBH_3$). The reaction proceeded under mild agitation for 24, 48 or 72 hours.

The yields of mono-PEGylated AAT under different reaction conditions were compared and the condition presenting the highest yield of mono-PEGylated AAT, the lowest yield of side products, and the shortest reaction time was chosen. In plasma-purified glycosylated AAT, a free cysteine is conjugated to the cysteine 232 residue and is described as a “cysteine cap” (Kolarich et al., 2006). This additional cysteine residue provides an additional N-terminal amino acid residue with an alpha-amino

group, which could react with the PEG-aldehyde in addition to the main polypeptide chain N terminal amino acid residue. To investigate the influence of the cysteine cap on N-terminal selectivity, the N-terminal PEGylation was carried out with or without a prior TCEP reduction step.

2.3 Purification of mono-PEGylated AAT

Mono-PEGylated AAT was purified by anion-exchange chromatography (AEC). The reaction mixtures were dialyzed against the starting buffer for FPLC (20 mM $N(CH_2CH_2OH)_3$, 5 mM NaCl, pH 7.5) for at least 6 hours (Spectra/Pro molecular porous membrane tubing MWCO 6-8 kDa, Spectrum, Belgium). The samples were filtered through a 0.22 µm polyvinylidene fluoride (PVDF) syringe-tip before loading to the FPLC column.

To optimize the reaction conditions, an anion exchange Resource Q1 column (GE Healthcare Bio-Science AB, Uppsala, Sweden) was used to purify the reaction mixture. A salt gradient elution was performed using the starting buffer 20 mM $N(CH_2CH_2OH)_3$, 5 mM NaCl, pH 7.5 and the elution buffer 20 mM $N(CH_2CH_2OH)_3$, 250 mM NaCl, pH 7.5. Absorbance at 280 nm was recorded during the purification. Chromatograms were recorded by UNICORN 5.2 software and the yield of PEGylated AAT was calculated based on the relative area under the peaks.

An anion exchange Mono Q 4.6/100 PE column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to purify the PEGylated AAT produced with the optimized conditions, using the same gradient elution as above. The collected fractions containing the mono-PEGylated AAT were pooled and concentrated using Vivaspin 15R sample concentrator (10,000 molecular weight cut-off, Sartorius, Stonehouse, Gloucestershire, UK) and stored in 10 mM NaH_2PO_4, 110 mM NaCl, pH 7.1 at 4 °C.

2.4 SDS-PAGE and Native PAGE

The purity of the purified AAT and PEGylated AAT was evaluated by SDS-PAGE using a gradient 4-20 % Mini-PROTEAN®TGX™ precast gel (Bio-Rad, Hercules, CA, USA). Samples were diluted with non-reducing or TCEP reducing 4X Laemmli Sample buffer (Bio-Rad, Hercules, CA, USA) and 3 µg of protein was loaded on each well. The Native PAGE was performed as described above but at 4°C instead of room temperature and without the presence of sodium dodecyl sulfate (SDS) in the electrophoresis buffer. The electrophoresis was performed for 1-2 h longer than usual to ensure a better separation for heavier protein components (PEG-AAT or the protease-inhibitor complex). The native page molecular markers were from SERVA Electrophoresis GmBH. The gels were stained by GelCode® Blue Stain Reagent (Thermo Fisher, Gent, Belgium) according to the manufactory instructions. PEG was stained using the barium iodide staining method based on Kurfürst's method(Kurfürst, 1992).

2.5 Dynamic light scattering (DLS)

The hydrodynamic diameter of AAT and PEGylated AAT was measured by DLS on a Zetasizer Ultra system (Malvern Panalytical Ltd, Malvern, UK). The PEG-maleimide, PEG-aldehyde and protein samples at 1 mg/ml in 10 mM NaH_2PO_4, 110 mM NaCl, pH 7.1 were filtered through a 0.22 µm PVDF syringe-tip prior to the DLS measurements. Observations were made at multiple angles. The scattering intensity data were transformed to volume distribution data and analyzed by Zetasizer Ultra software.

2.6 Electrospray-ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF)

The mass spectrometry analysis was performed according to Kolarich et al (Kolarich et al., 2006). The detailed methods are available in the supplementary document.

2.7 Elastase inhibition assay

The biological activity of AAT and PEGylated AAT was evaluated by a modified elastase inhibition assay according to Bieth(Lestienne and Bieth, 1978). Briefly, at 37 °C, 0.034 µM hNE was pre-incubated with 0.3 µM substrate N-Succinyl-Ala-Ala-Ala-pNitroanilide (Tris-NaCl buffer, pH 7.5) in a 96-well plate for 20 min. Afterwards, AAT or PEG-AAT was added to the mixture at a protein:hNE molar ratio of 0.3:1, 0.6:1, 1:1, 1.3:1, 1.6:1 and 2:1 and incubated for 40 min. During the incubation, the absorbance at 410 nm of each sample was recorded every 1 min. The absorbance at 410 nm increases linearly with incubation time. The increment of the absorbance at 410 nm of each sample (ΔODsp) after adding the unconjugated or PEGylated AAT was used to calculate the relative hNE activity. The ΔOD410 of the sample in absence of AAT (ΔODctr) represented the full activity of hNE. The relative hNE activity was calculated as follows: relative hNE activity (%) = ΔODsp / ΔODctr × 100.

2.8 Inhibitor-protease complex formation

Unconjugated AAT and PEG-AAT were incubated with hNE (on ice) in NaOAc-NaCl, pH 5, or with PR3 (at room temperature) in HEPES buffer, pH 7.4 for 5 min. The molar ratio of AAT or PEG-AAT to the protease was 1:1. The mixture was then subjected to native PAGE and SDS-PAGE, followed by a silver stain using Pierce™ Silver Stain Kit (Thermo Fisher, Gent, Belgium) to visualize the protein and protein-protease complex.

To identify the protein-protease complex in SDS-PAGE, a Western Blot method was performed. Following the separation by SDS-PAGE, the proteins were electroblotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). In this step, AAT and PEG-AAT showed different migration rate from the gel to the membrane. To ensure the successful transfer of PEG-AAT, the

electroblotting conditions were optimized for PEG-AAT according to the manufacturer instructions. After blocking in 5 % non-fat milk at room temperature for 1 hour, the membrane was incubated in a 1:5,000 dilution rabbit anti-hNE conjugated to HRP (ABIN2753573, CUSABIO, Kampenhout, Belgium) at 4 °C overnight. The membrane was washed with phosphate-buffered saline containing 0.1 % Tween™ 20 (PBST) for three times, the bound antibody was detected by chemiluminescence using Pierce™ ECL Western Blotting Substrate (Thermofisher, Gent, Belgium) according to the manufacturer manual.

2.9 Stability of AAT and PEG-AAT during long-term storage

The purified unconjugated AAT and PEGylated AAT were stored in 10 mM NaH_2PO_4, 110 mM NaCl, pH 7.1 at 4 °C for 24 weeks. Protein samples were prepared in separate Eppendorf tubes and sealed by PARAFILM® M (Sigma-Aldrich, Overijse, Belgium). The content of monomers, aggregates and fragments of the protein samples were analyzed by UV absorbance and SEC, using a Superose 6 increase 5/150 GL column (GE Healthcare Europe GmbH, Diegem, Belgium) after 2, 4, 8, 12, 16, 20, 24 weeks of storage. The monomer content of each sample was monitored by SEC and calculated using the area under the curve of peaks in SEC chromatograms. The activity of AAT and PEG-AAT was evaluated by the elastase inhibition assay as described in 2.7.

2.10 Cytotoxicicy of AAT and PEG-AAT

Murine alveolar macrophages MHS (ATCC® CRL-2019 TM) were cultured at 37 ◦C and 5 % CO_2 in RPMI-1640 medium (Thermofisher, Gent, Belgium). The medium was supplemented with 10 % Foetal Bovine Serum (FBS, Thermofisher, Gent, Belgium) and 2-mercaptoethanol (2-ME, Thermofisher, Gent, Belgium) at a final concentration of 0.05 mM. Cells were subcultured twice a week and maintained within the passage number between 10 – 20. MHS cells were seeded in 96-well plates (Thermofisher, Gent, Belgium) at a cell density of 2.5×10^4 cells/well and cultured for 24 h before the exposure to the tested proteins. AAT or PEG-AAT were incubated with MHS at a final concentration of 0.02 – 2 µg/ml, the control cells were incubated with complete RPMI medium with the addition of the same volume of AAT storage buffer without the protein. After 48 hour-exposure to AAT or PEG-AAT, the culture medium was replaced with complete RPMI medium with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After 3 hours incubation, 100 µl of lysis buffer (10 % SDS, 0.01 N HCl) were added to each well. The plates were slowly shaked for 4 hours. The absorbance of each well at 560 nm was measured by SpectraMax i3 (Molecular Devices, CA, USA). The cell viability was calculated by the following equation: %cell viability = ODsp/ODctr × 100 %.

2.11 Statistics

GraphPad Prism (GraphPad Software, USA) was used to infer statistical differences. In elastase inhibition assays, the values were compared by unpaired multiple T test followed by Holm-Sidak method. In MTT assays, the results were compared by One-Way ANOVA using Dunnett comparison test (* $p<0.05$).

3 Results

3.1 Production and purification of PEG-AAT

Thiol PEGylation was conducted using linear PEG-maleimide (PEGmal) of 30 kDa or 40 kDa and 2-armed PEGmal of 40 kDa (Figure 1). Anion exchange chromatography (AEC) was used to purify PEGylated AAT. As shown in Figure 2, the first eluted peak corresponds to the PEGylated AAT and the second peak to unmodified AAT. A SDS-PAGE of the fractions confirmed the PEGylation degree of PEGylated AAT and no multi-PEGylated products were found (Supplementary Figure S2). The TCEP-reduction step is essential for thiol PEGylation. Omitting the reduction step resulted in a low yield of PEGylated AAT (Figure 2 A).

The molar ratio of PEG to AAT and the AAT concentration were varied to improve the yield of mono-PEGylated AAT using 2-armed 40 kDa PEG-maleimide. At the same AAT concentration (10 mg/ml), doubling the PEG:AAT molar ratio (from 2:1 to 4:1) increased the yield of mono-PEGylated AAT by 1.5-fold (Figure 2 B, bottom and middle curves). At the same PEG:AAT molar ratio of 4:1, doubling the concentration of AAT (from 10 mg/ml to 20 mg/ml) resulted in a 1.6-fold improvement of mono-PEGylated AAT (Figure 2 B, middle and upper curves). Increasing the reaction time from 1 h to 8 h did not result in higher yield (Supplementary Figure S3), indicating that at 1 h, the reaction already reached completion. The conditions showing the highest yield of mono-PEGylated AAT (20 mg/ml AAT, PEG:AAT 4:1, 1 h reaction time) were selected to produce PEG-AAT by thiol PEGylation.

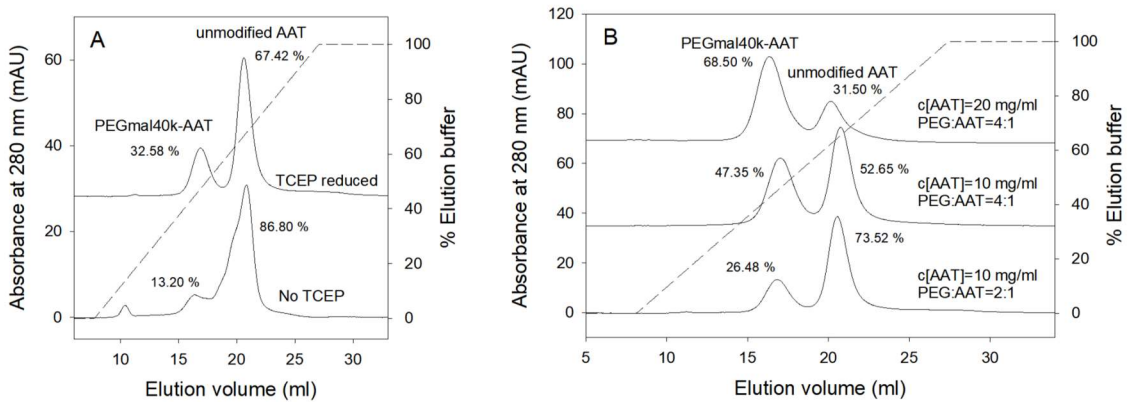


Figure 2. Optimization of thiol PEGylation. Two-armed 40 kDa PEG-maleimide was used. The reaction was at room temperature, pH 7.1, for 24 h, with different concentrations of AAT or PEG. A) Anion exchange chromatograms of non-reduced and TCEP-reduced thiol PEGylation. The concentration of AAT was 5 mg/ml, the molar ratio PEG:AAT 2:1. In TCEP-reduced PEGylation, the AAT was reduced by 4-fold molar excess of TCEP for 1 h prior to the PEGylation reaction for 1 h. Without the prior TCEP reduction, the yield of thiol PEGylation was low. B) Impact of PEG:AAT molar ratio and protein concentration on thiol PEGylation. The AAT was reduced by 4-fold molar excess of TCEP prior to the reaction for 1 h. Increasing PEG:AAT molar ratio or AAT concentration both resulted in higher PEGylation yield.

N-terminal PEGylation was performed using linear PEG-aldehyde (PEGald) of 30 kDa or 40 kDa and 2-armed PEGald of 40 kDa. The full UV spectrum of AAT shifted to higher absorbance values when decreasing the buffer pH. A significant absorbance at the wavelength of 300 – 350 nm (Figure 3 A) where proteins do not absorb indicates the formation of protein aggregates. Due to the instability of AAT in the pH range of 4 to 5.5, pH 6.0 was chosen to perform the N-terminal PEGylation. In the chromatograms of N-terminal PEGylation, three peaks were observed and identified by SDS-PAGE (Supplementary Figure S4) as di-PEGylated, mono-PEGylated and unmodified AAT (Figure 3 B, C). The peak of di-PEGylated AAT showed considerable overlap with the mono-PEGylated product.

The effect of protein concentration and PEG:AAT molar ratio on the degree of PEGylation and yield were investigated. As shown in Figure 3B, at the same PEG:AAT molar ratio (2:1), doubling the protein concentration from 5 mg/ml to 10 mg/ml increased the yield of mono-PEGylated AAT (by 1.8-fold) but also di-PEGylated AAT (by 2.2-fold). At 5 mg/ml, PEG:AAT molar ratios of 2:1, 4:1 and 8:1 were tested for 24-hour reaction time. As shown in Figure 3C, increasing the PEG amount led to an increased yield of mono- and di-PEGylated AAT. The yield of mono-PEGylation increased slightly when increasing the PEG:AAT molar ratio from 4:1 to 8:1, while the di-PEGylation increased tremendously. Interestingly, the yield of mono-PEGylated AAT was similar between PEG :AAT 4 :1, AAT 5mg/ml and PEG;AAT 2:1, AAT 10mg/ml, where the PEG concentration was the same. Increasing the reaction time from 24 h to 72 h did not result in higher mono-PEGylation. However, it slightly increased the di-PEGylation yield (Supplementary Figure S3). The experimental condition of

10 mg/ml AAT, PEG:AAT 2:1, reaction time 24 h were kept to produce the mono-PEGylated AAT as it presented decent yield of mono-PEGylated AAT and lower yield of side products.

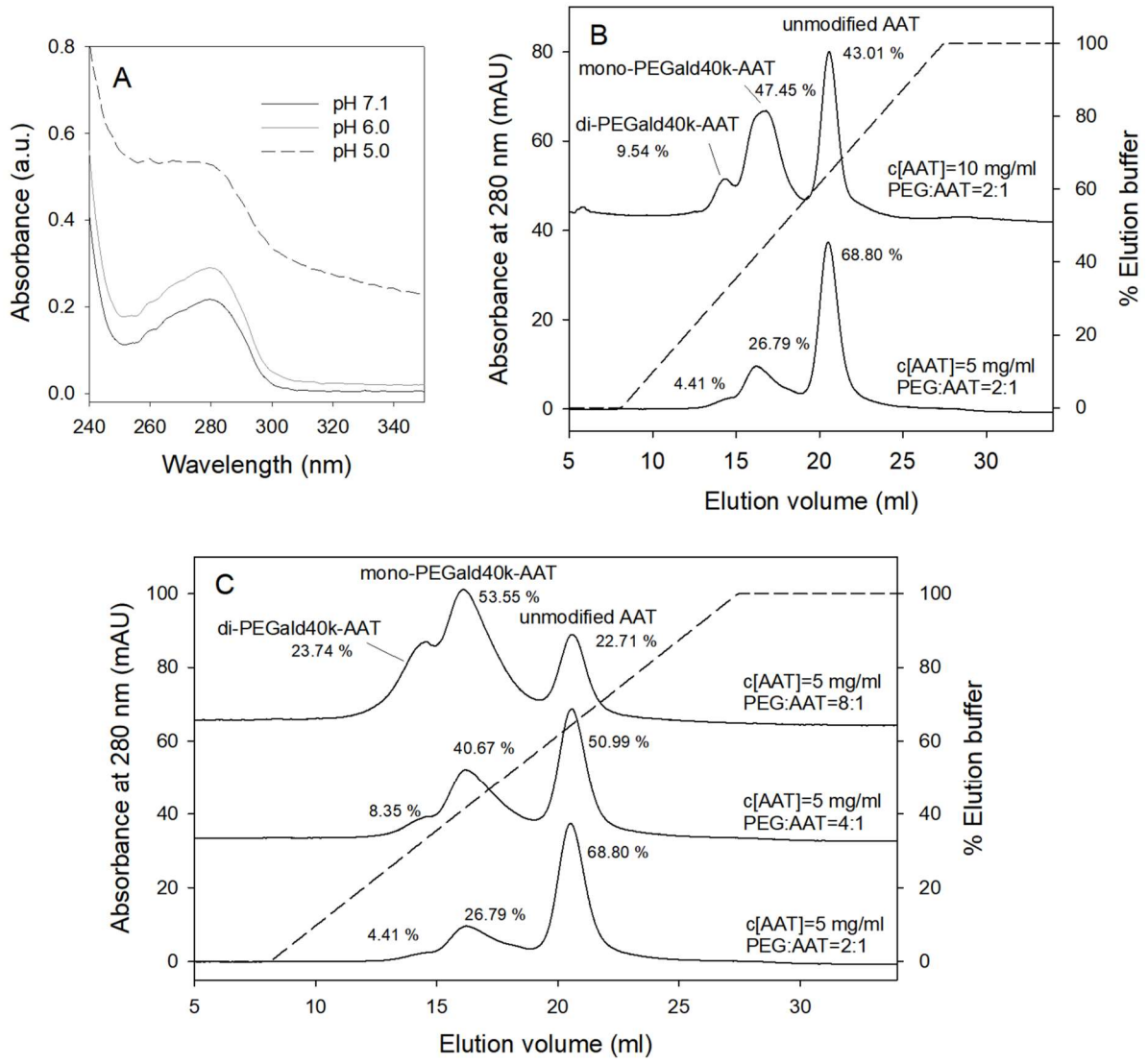


Figure 3. Optimization of N-terminal PEGylation. A) UV spectrum of AAT at different pH. At pH 5, the absorbance of AAT at 310 nm significantly increased, indicating the formation of insoluble aggregates. pH 6 was chosen for N-terminal PEGylation. B) Anion exchange chromatograms showing the effect of protein concentration on N-terminal PEGylation and C) the effect of PEG:AAT molar ratio on N-terminal PEGylation. Reactions were performed at pH 6.0 for 24 h at room temperature. Increasing PEG:AAT molar ratio or AAT concentration both led to higher PEGylation yield. However, when increasing the PEG:AAT molar ratio from 4:1 to 8:1, the mono-PEGylation yield increased slightly while the di-PEGylation yield increased tremendously.

To have a better separation/resolution, a Mono Q 4.6/100 PE column was used to purify the mono-PEGylated AAT as it has smaller particle size (10 µm) than Resource Q1 column (15 µm). Figure 4 shows the chromatograms of thiol and N-terminal PEGylation reaction mixtures, which both show better resolution than in the Resource Q1 column. The overlap between the peaks of di- and mono-

PEGylated AAT was reduced for N-terminal PEGylation. Interestingly, in N-terminal PEGylation, the major peak of mono-PEGylated AAT showed a split peak. This might indicate a heterogeneity of the mono-PEGylated product. The yield of each PEGylation reaction is listed in Table 1.

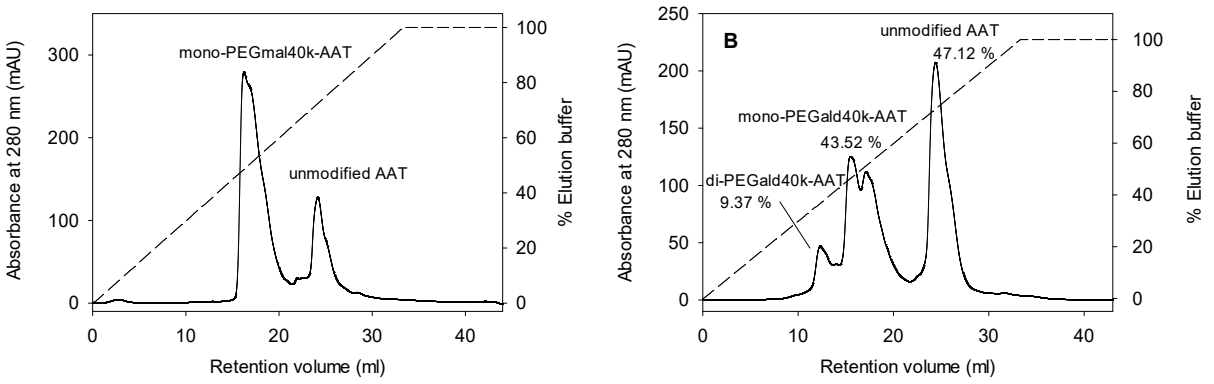


Figure 4. Anion exchange chromatograms of the PEGylation reaction mixtures using Mono Q 4.6/100 PE column. A) Thiol PEGylation; B) N-terminal PEGylation. Mono Q 4.6/100 PE column shows better resolution than the Resource Q1 column.

Table 1. PEGylation conditions and mono-PEGylation yield.

PEGylation	PEG	pH	AAT	Reducing agent	Molar ratio [PEG:AAT]	Reaction time	mono-PEGylation Yield (%)[a]
Thiol	Linear, 30 kDa	7.1	20 mg/ml	1.8 mM TCEP	4:1	1 h	63
	Linear, 40 kDa						66
	2-armed, 40 kDa						64
N-terminal	Linear, 30 kDa	6	10 mg/ml	23 µM $NaBH_3CN$	2:1	24 h	44
	Linear, 40 kDa						40
	2-armed, 40 kDa						44

[a]The yield of mono-PEGylated AAT was calculated by measuring the relative area under the peak corresponding to mono-PEGylated AAT in anion exchange chromatograms to the total surface areas of all peaks. Each reaction was performed at least twice, and the average yield is presented in the table.

The purified mono-PEGylated AAT compounds were visualized on the gel (Figure 5). The unmodified AAT (52 kDa) migrated to ~50 kDa while the PEGylated AAT showed higher apparent molecular weight (MW) compared with the theoretical MW. Linear PEGmal30k-AAT and linear PEGmal40k-AAT migrated to a MW of ~130 and ~150 kDa, respectively. 2-armed PEGmal40k-AAT migrated to even higher MW – approximately 200 kDa. The bands of linear PEGald30k-AAT and linear PEGald40k-AAT splited into two separated bands (Figure 5B), which indicates an heterogeneity of these two products. This result is in line with the chromatogram of the reaction mixture of N-terminal PEGylation (Figure 4 B). We suspected that the two separated bands on the gel resulted from PEG conjugation to the alpha-amino groups of the N-terminal glutamate residue and the additional cysteine conjugated to the cysteine 232 residue. To test our hypothesis, we performed the N-terminal

PEGylation using linear 30 kDa PEG-aldehyde with a prior TCEP-reduction step to remove the additional cysteine cap on the cysteine 232 residue. The mono-PEGylated products from both reactions (with or without a prior TCEP-reduction) were visualized on the gel. As shown in Figure 5C and D, without the prior TCEP-reduction, the produced mono-PEGylated AAT was degraded, and free AAT and free PEG were released upon incubation with TCEP. While with a TCEP-reduction before PEGylation, the mono-PEGylated product was stable to further reduction.

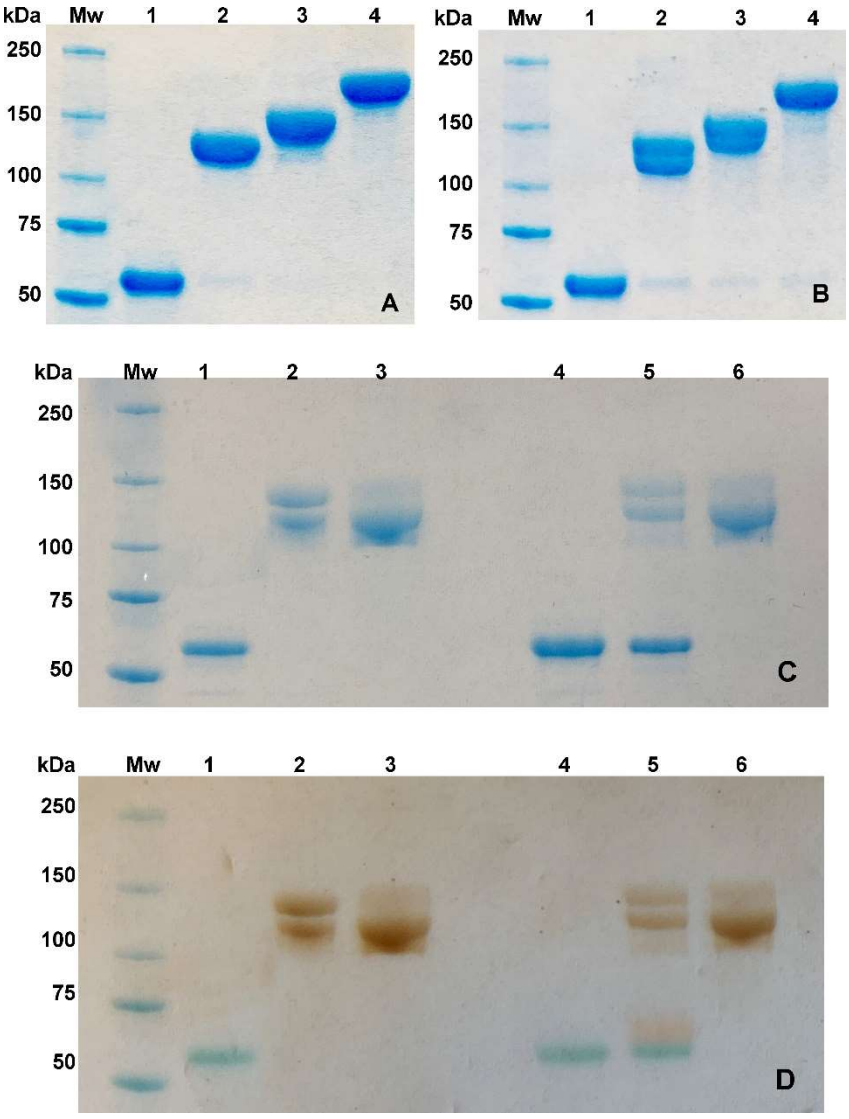


Figure 5. SDS-PAGE of AAT and PEGylated AAT stained by Coomassie blue. A) Thiol PEGylation; B) N-terminal PEGylation. Lane 1: unconjugated AAT; lane 2-4: linear PEG30k-AAT, linear PEG40k-AAT and 2-armed PEG40k-AAT. C) SDS-PAGE of purified AAT and linear PEGald30k-AAT synthesized without or with a prior TCEP-reduction, stained by coomassie blue. Lane 1-3: purified AAT; linear PEGald30k-AAT; linear PEGald30k-AAT, TCEP-reduced before PEGylation; Lane 4-6: the same as lane 1-3 but after incubation with TCEP before loading on the gel. D) the same gel as in C but stained by barium iodine (PEG staining). With prior TCEP reduction, the mono-PEGylated AAT was stable to further reduction. Without the prior TCEP reduction, the mono-PEGylated AAT was degraded when exposed to reducing agent and released the free PEG and AAT.

3.2 Physico-chemical characterization of PEG-AAT

The hydrodynamic size of PEG-maleimide, PEG-aldehyde and PEGylated AAT was analyzed by dynamic light scattering (DLS). As shown in Supplementary Table S1, PEG chains with the same molecular weight shared the same hydrodynamic diameter independently of PEG architecture. The size of unmodified AAT was 6.2 ± 0.1 nm. Upon PEGylation with linear PEG30k, the hydrodynamic size increased to 10.8 ± 0.4 nm (thiol PEGylation) and 9.6 ± 0.1nm (N-terminal PEGylation). Linear PEGmal40k-AAT and 2-armed PEGmal40k-AAT shared a similar size.

Table 2. Hydrodynamic diameter of unmodified AAT and PEGylated AAT measured by DLS

Macromolecules	**Averaged size (nm)**	**% volume of the main peak**	**PDI**
AAT	6.2 ± 0.1	100	0.07 ± 0.01
Linear PEGmal30k-AAT	10.8 ± 0.4	100	0.20 ± 0.03
Linear PEGmal40k-AAT	12.9 ± 0.2	99.99	0.20 ± 0.02
2-armed PEGmal40k-AAT	12.5 ± 0.7	100	0.10 ± 0.02
Linear PEGald30k-AAT	9.6 ± 0.1	99.99	0.20 ± 0.01
Linear PEGald40k-AAT	11.9 ± 0.3	100	0.10 ± 0.01
2-armed PEGald40k-AAT	13.0 ± 0.2	100	0.20 ± 0.03

An intact protein mass spectrometry analysis was performed to determine the molecular weight of AAT and PEG-AAT. However, for both AAT and PEG-AAT, we failed to obtain reproducible results with high resolution. This might be due to the three glycosylation sites on the native protein, leading to a highly complex m/z charge distribution. Digested protein samples were analyzed by LC-MS-MSMS and a local database containing the full sequence of AAT was used for peptide matching. The peptide coverage of unconjugated AAT, thiol PEGylated AAT (linear PEGmal30k-AAT) and N-terminal PEGylated AAT (linear PEGald30k-AAT) was 71 %, 40 % and 31 %, respectively (Supplementary Table S2). The peptide sequence containing the cysteine 232 (residue 224 – 244) was found in unconjugated AAT and linear PEGald30k-AAT but not in linear PEGmal30k-AAT, indicating that the linear 30 kDa PEG-maleimide was conjugated to this site. In unconjugated AAT, the N-terminal

sequence was weakly detected. This sequence was not found in linear PEGald30k-AAT (Supplementary Table S3).

3.3 Biological activity, stability and biocompatibility of PEG-AAT

The elastase inhibitory activity of thiol PEGylated or N-terminal PEGylated AAT was measured and compared to unconjugated AAT (Figure 6). The PEGylated AAT fully preserved the *in vitro* anti-protease activity of the unmodified protein. A complete inhibition of elastase activity was achieved with an inhibitor:hNE molar ratio of 1:1 in both PEGylation chemistries.

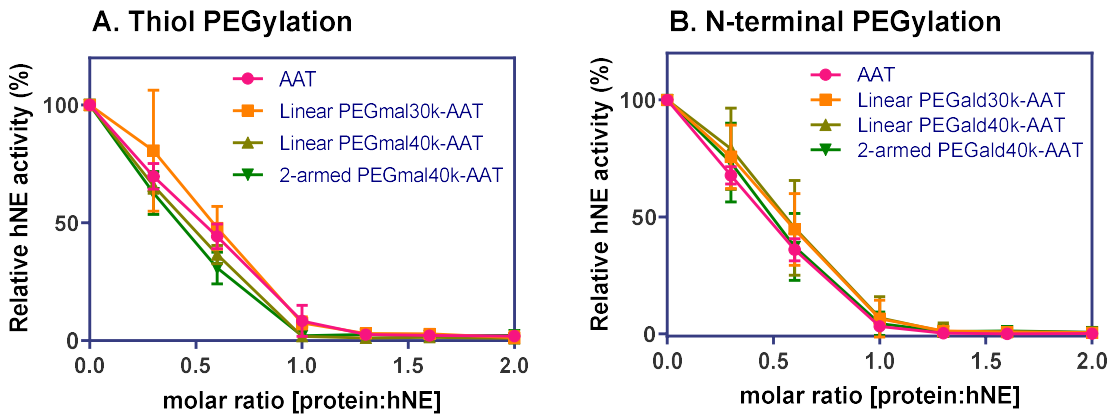


Figure 6. The elastase inhibition activity of unmodified AAT and PEGylated AAT. PEG-AAT showed the same activity as unmodified AAT. A) Thiol PEGylation; B) N-terminal PEGylation. Multiple T test and Holm-Sidak method were performed, and no statistical difference was found ($p>0.05$).

Native AAT neutralizes proteases by forming a covalent acyl AAT-protease complex (Duranton et al.; Duranton and Bieth, 2003; James and Cohen, 1978). The complex AAT formed with hNE and PR3 was separated by native PAGE and SDS-PAGE and visualized by silver staining. Silver binds to the N-terminal or side chain amino groups. The presence of basic amino acids in proteins is essential for the success of silver staining. Unforturnately, hNE comprises no lysine (Heck et al., 1985). Therefore, elastase is difficult to visualize by silver staining. As shown in Figure 7A and B, bands at MW higher than the free AAT compounds were observed on the gel separated by native PAGE in both unmodified AAT and thiol PEGylated AAT incubated with PR3 or hNE. This indicates that PEG-AAT preserved the ability to form the protease-inhibitor complex with PR3 and hNE. Interestingly, with SDS-PAGE, bands appeared at higher MW than the unmodified AAT but at lower MW than the PEGylated AAT following incubation with PR3 and hNE (Figure 7C, D). An hNE-specific western blot was performed to identify the AAT-hNE and PEG-AAT-hNE complexes in the SDS-PAGE. As shown in Figure 7 D and E, the faint band above the band of the unmodified AAT and the bands with lower MW than the PEG-AAT contained hNE. This suggests that the PEG-AAT-hNE complex migrated faster in the gel

matrix than PEG-AAT. PEG-AAT and the PEG-AAT-hNE complexes are more difficult to transfer to the nitrocellulose membrane than hNE, AAT and AAT-hNE. When an appropriate amount of PEG-AAT and PEG-AAT-hNE complex were electroblotted to the membrane, hNE, AAT and hNE-AAT already penetrated the membrane. This is the reason why we did not detect hNE in WB and the WB band of AAT-hNE was faint.

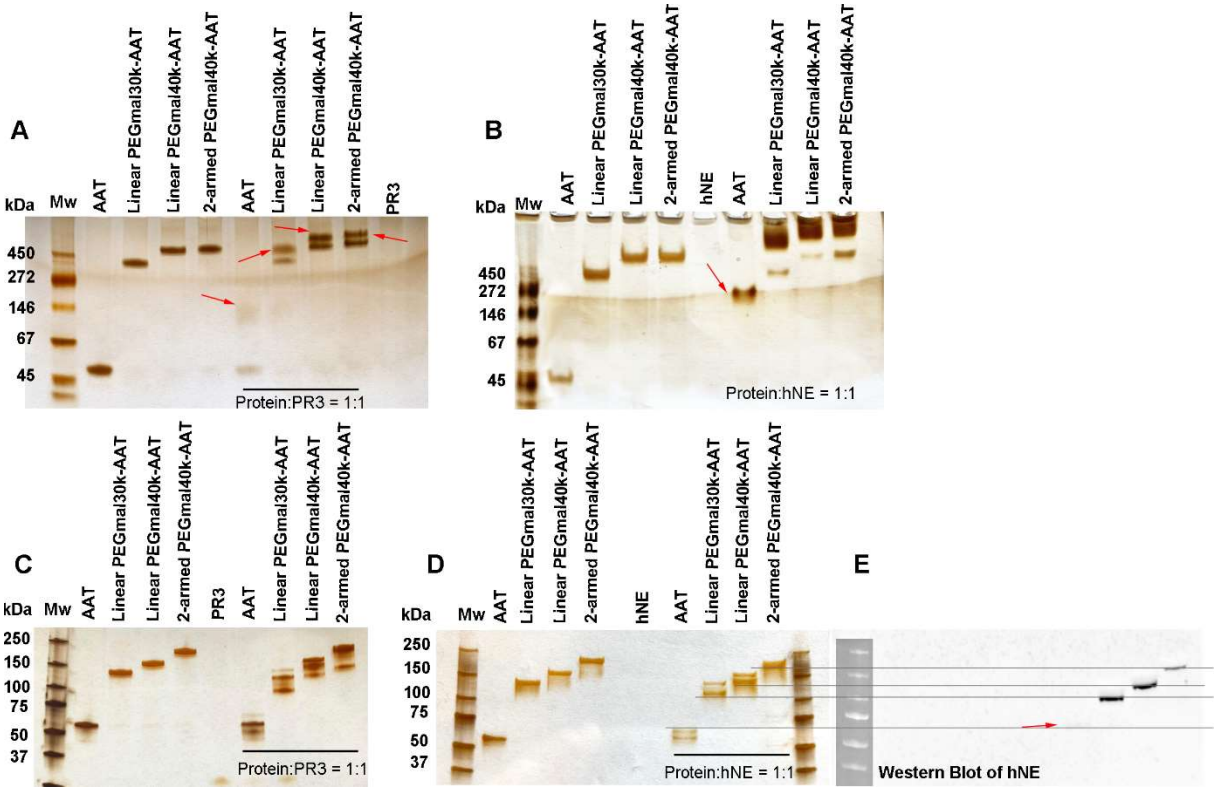


Figure 7. A) Native PAGE of purified and thiol PEGylated AAT incubated with PR3, molar ratio [protein:PR3] = 1:1. The red arrows on the gel indicate the AAT-PR3 complex; B) Native PAGE of purified and thiol PEGylated AAT incubated with hNE at a 1:1 protein:hNE molar ratio; AAT and PEG-AAT were able to form the protease-inhibitor complex with PR3 or hNE. C) SDS-PAGE of purified AAT and thiol PEGylated AAT incubated with PR3 at a 1:1 protein:PR3 molar ratio. D) SDS-PAGE of purified AAT and thiol PEGylated AAT incubated with hNE at a 1:1 protein:hNE molar ratio; E) Western Blot of hNE in AAT-hNE or PEG-AAT-hNE complexes. The original SDS-PAGE gel of E) was to the same as the gel shown in D). The bands with lower molecular weight than PEG-AAT contained hNE, indicating that the hNE-PEG-AAT complex migrates faster than PEG-AAT in SDS-PAGE.

Although PEG-AAT presents the same biological activity as AAT, we were concerned about the long-term stability of the conjugates. Therefore, the activity of AAT and PEGmal-AAT stored in solution at 4°C was monitored over a period of 24 weeks. The possible aggregation was determined by UV absorbance and SEC. During the 24 weeks of storage, the UV spectrum of AAT or PEG-AAT showed no significant change (Figure S6), indicating that there was no formation of insoluble aggregates. The monomer content of each sample monitored by SEC remained stable during 24 weeks of storage

(Figure 8A). The monomer content of all protein samples on the last day of storage was not statistically different from the one on the first day of storage ($p>0.05$). Figure 8B illustrates the hNE inhibition capacity of AAT and PEG-AAT at a protein:hNE molar ratio 1:1. The elastase inhibitory activities of AAT and 2-armed PEGmal40k-AAT showed no significant changes after 24 weeks of storage, while the activity of linear PEGmal-AAT decreased ($p<0.01$). These results indicate that AAT and 2-armed PEGmal40k-AAT are stable during 24 weeks of storage, while linear PEGmal-AAT might lose some biological activity.

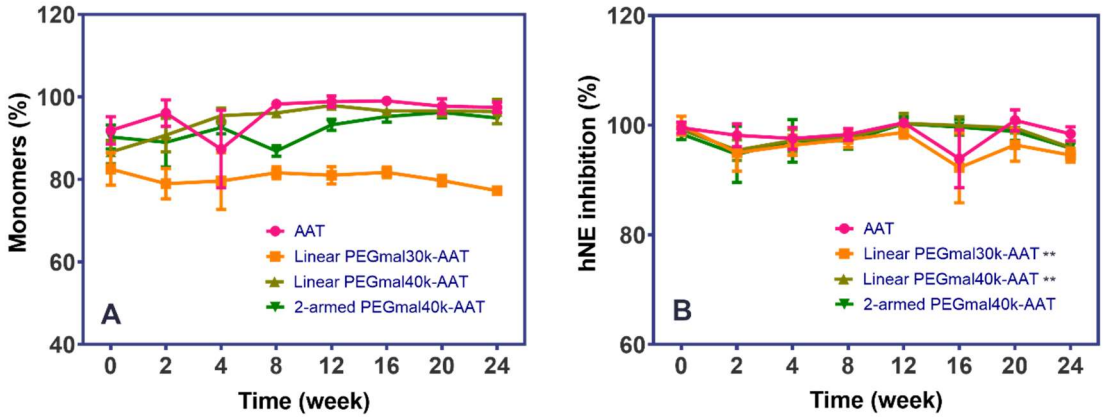


*Figure 8. A) The protein monomer content of AAT and PEGmal-AAT during storage. The percentage of monomer was calculated by the area under the curve of SEC chromatograms. No significant difference was found between the results of the last day and the first day of storage. B) The hNE inhibition capacity of AAT and PEG-AAT (protein:hNE molar ratio was 1:1) during storage. The inhibitory activities of linear PEGmal30k-AAT and liner PEGmal40k-AAT on the last day of storage were significantly different from those on the first day of storage. Statistical difference was determined by one-way ANOVA using Dunnett comparison test (n=3, ** $p<0.01$).*

The cytotoxicity of AAT and PEG-AAT was determined by the MTT assay. MHS cells were incubated with different concentrations of AAT or PEG-AAT for 48 hours. As shown in Figure S7, AAT and PEG-AAT did not show cytotoxicity in MHS cells. After 48 hours exposure to 2 µg/ml AAT, 2-armed PEGmal40k-AAT or 2-armed PEGald40k-AAT, the cell viability was 101 ± 5 %, 100 ± 3 % and 101 ± 7 %, respectively.

4 Discussion

In this article, we successfully PEGylated AAT at two different sites: the N-terminus and the cysteine 232. In both PEGylation chemistries, a prior reduction step was required and the AAT concentration and PEG:AAT molar ratio influenced the yield of mono-PEGylated AAT. An acidic reaction pH was essential for N-terminal selectivity in N-terminal PEGylation but the pH could not be chosen too

acidic because of AAT instability. PEGylation preserved AAT biological activity and its capacity to form an inhibitor-protease complex.

The results of the hNE inhibition assay confirmed the full preservation of the biological activity of AAT upon PEGylation on either the N-terminus or the cysteine 232, which is consistent with the capacity of the PEGylated protein to form a protease complex with both PR3 and hNE. This result is in line with Cantin et al (Cantin et al., 2002), who demonstrated that thiol PEGylation (using 20 and 40 kDa PEG) of rhAAT did not affect its *in vitro* elastase inhibition activity. However, protein PEGylation can actually result in a significant loss of biological activity of therapeutic proteins. Granulocyte colony-stimulating factor (G-CSF), a 19 kDa glycoprotein, showed reduced *in vitro* biological activity by 3 - to 50-fold after PEGylation, depending upon the number and sizes of the attached PEG molecules(Kumari et al., 2020; Rosendahl et al., 2005). However, G-CSF conjugated to 20 kDa PEG was later developed as Neulasta®, a marketed medicine to treat neutropenia, as it provides prolonged half-life, which offsets the loss of activity and leads to an overall better therapeutic value. Similarly, Pegasys®, a PEGylated interferon-α-2a, preserved only 7 % of the *in vitro* activity of the unconjugated cytokine but showed an excellent *in vivo* efficacy (Veronese and Mero, 2008). Therefore, the full preservation of the biological activity of AAT after PEGylation *in vitro* is promising and PEGylation might improve its *in vivo* activity even more.

Interestingly, we have noticed that the PEG-AAT-hNE complex migrated more slowly in native PAGE but faster in SDS-PAGE compared to the free PEG-AAT. Taking linear PEGmal30k-AAT as an example, the protein part of the conjugate is 52kDa, which comprises 63 % of the total MW of 82kDa. While after forming a complex with hNE, the molecular weight of the protein part in the complex (AAT+ hNE) is 81.5 kDa which accounts for 73 % of the total MW of 111.5kDa of the complex. The increase in the protein proportion in the complex appears to counterbalance the slowdown due to PEG in the electrophoretic migration rate in SDS-PAGE, probably because the negativey-charged SDS binds the protein rather than the PEG moiety.

Thiol PEGylation is commonly performed at slightly acidic or neutral pH(Dozier and Distefano, 2015) while N-terminal PEGlyation is widely performed at pH 4.5 – 6 for better selectivity(Hu and Sebald, 2011; Wu et al., 2013; Zhao et al., 2012). The selectivity of N-terminal PEGylation is achieved by taking advantages of the different pKa values of the ε-amino residue (9.3 – 9.5) of lysines and the α-amino residue (7.6 – 8.0) of the N-terminal residue (Pasut and Veronese, 2012). At acidic pH, conjugation by nucleophilic attack favors the α-amino groups, which are less protonated compared with the ε-amino groups in the lysines. A reduction step was necessary before thiol PEGylation as the cysteine 232 in AAT is linked to another cysteine via a disulfide bond (Kolarich et al., 2006), making the -SH group inaccessible. Omitting the prior reduction step resulted in a low yield (14 %) for thiol PEGylation when conjugating PEG-maleimide directly to native AAT. Moreover, the additional cysteine cap on the cysteine 232 residue provides another free N-terminal residue for N-terminal

PEGylation. With no prior reduction, PEG-aldehyde reacted with both the amino groups on the cysteine cap and the N-terminal amine and this heterogeneous product was unstable in reducing conditions and partly degraded into free AAT and free PEG. In both PEGylation chemistries, increasing the AAT concentration or the PEG:AAT molar ratio increased the yield of mono-PEGylation. In both cases as well, prolonging the reaction time beyond a threshold did not increase the yield of mono-PEGylation as the yield of mono-PEGylation reached a plateau at 1 h (thiol PEGylation) and 24 h (N-terminal PEGylation). Between the two PEGylation chemistries, we favor thiol PEGylation for its shorter reaction time, milder reaction conditions, easier purification (no di-PEGylated products) and higher yield. Based on Guichard et al (Guichard et al., 2021), we expect that the conjugation to the largest PEG already used clinically (2-armed 40kDa PEG) will best lead to prolonged AAT half-life and improved AAT therapeutic efficacy in vivo.

The PEGylation degree was mainly characterized by SDS-PAGE and DLS and confirmed the mono-PEGylation of the products. The intact protein MS analysis of AAT was unsuccessful due to its heterogeneous glycosylation profile(Bengtson et al., 2016; Chen et al., 2011) (Bengtson et al., 2016; Chen et al., 2011). In peptide mapping, the N-terminal sequence was weakly detected in unconjugated AAT while completely absent in N-terminal PEGylated AAT. Mills et al separated 6 subspecies of AAT by isoelectric focusing and two-dimension SDS-PAGE and showed that two lacked the five N-terminal amino acid (EDPQG)(Mills et al., 2001). In addition, the N-terminal sequence showed a weak signal and sometimes was undetectable, especially in di-glycosylated AAT(Mills et al., 2003). These results highlight the difficulties to detect the N-terminal sequence of AAT in MS analysis. Therefore, due to the low peptide coverage as well as the technical difficulty in detecting the N-terminus of AAT, it is not fully proven that the PEG-aldehyde was indeed conjugated to the N-terminus. On the other hand, the sequence containing the cysteine 232 residue was detected in both native AAT and N-terminal PEGylated AAT, but not in thiol PEGylated AAT, which confirms that PEG-maleimide was successfully conjugated to Cys232 in thiol PEGylation and the TCEP reduction step prior to the N-terminal PEGylation prevented the conjugation to the free cysteine cap.

We produced mono-PEGylated AAT with preserved biological activity with the goal to increase the half-life of AAT following systemic or pulmonary administration. Besides the potential benefits from PEGylation, limitations of PEGylation have also been reported (Rondon et al., 2021). Anti-PEG antibodies have been detected in patients receiving PEGylated therapeutics and some of the patients displayed accelerated blood clearance or allergic reactions. Severe immunological response induced by PEGylated proteins in patients has been strongly associated with the immunogenecity of the carrier protein (Zhang et al., 2016). PEGylated non-human enzymes such as uricase (porcine enzyme) and asparaginase (*E coli* enzyme), generated severe issues because of anti-PEG antibodies, while some other PEGylated proteins were safe. In our case, we PEGylated human plasma-purified glycosylated AAT, which in theory would not exhibit high immunogenecity in patients. However, the safety of

PEGylated AAT should still be fully studied. It has been reported that anti-PEG antibodies might cause rapid clearance of the PEGylated protein (Rondon et al., 2021). However, the clinical outcome of these effects is difficult to predict as it will depend on the levels of antibodies generated. To conclude, PEGylated AAT needs to be thoroughly characterized for its immunogenecity, safety, and biodistribution before considering it for clinical use.

5 Conclusion

PEGylation of AAT with linear 30 kDa, 40 kDa and 2-armed 40 kDa PEG was achieved by N-terminal and thiol PEGylation with satisfactory yields of mono-PEGylated products. The mono-PEG-AAT preserved the capacity to form an inhibitor-protease complex and the full biological activity of unconjugated AAT. These results support further research on PEG-AAT and in particular pharmacokinetic and therapeutic efficacy studies in preclinical in vivo models following both inhalation and intravenous injection.

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