Protein Modification Hot Paper

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Site-Specific Conjugation of a Selenopolypeptide to Alpha-1-antitrypsin Enhances Oxidation Resistance and Pharmacological Properties

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Abstract: *Human alpha-1-antitrypsin (A1AT), a native serine-protease inhibitor that protects tissue damage from excessive protease activities, is used as an augmentation therapy to treat A1AT-deficienct patients. However, A1AT is sensitive to oxidation-mediated deactivation and has a short circulating half-life. Currently, there is no method that can effectively protect therapeutic proteins from oxidative damage in vivo. Here we developed a novel biocompatible selenopolypeptide and site-specifically conjugated it with A1AT. The conjugated A1AT fully retained its inhibitory activity on neutrophil elastase, enhanced oxidation resistance, extended the serum half-life, and afforded long-lasting protective efficacy in a mouse model of acute lung injury. These results demonstrated that conjugating A1AT with the designed selenopolymer is a viable strategy to improve its pharmacological properties, which could potentially further be applied to a variety of oxidation sensitive biotherapeutics.*

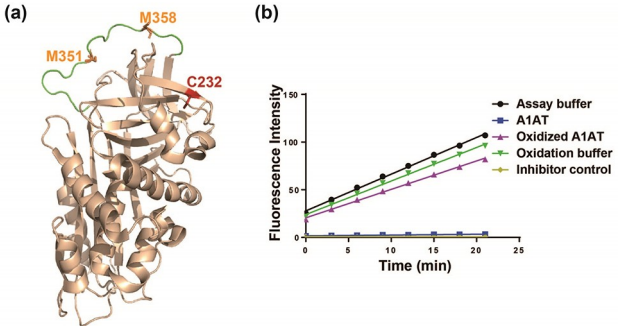

Figure 1. Characterization of purified A1AT. a) Structure of A1AT. Reactive center loop is colored in green, Met358 and Met351 are colored in orange, Cys232 is colored in red. b) Inhibitory activities of A1AT and oxidized A1AT, measured by Neutrophil Elastase inhibitor Screening Kit.

Human alpha-1-antitrypsin (A1AT) is a member of the serpin superfamily and one of the most abundant endogenous serine-protease inhibitors. It is secreted primarily by hepatocytes to the circulation to limit tissue damage by protease, such as neutrophil elastase (NE), in the presence of inflammation.[1] There is a reactive center loop (RCL, positions 357–366) that protrudes from the A1AT surface which reacts with the active serine, resulting in RCL cleavage, conformational change, and formation of an inactive covalent serpin-protease complex (Figure 1a).[2] A1AT deficiency (A1ATD) is a common hereditary disorder affecting approximately 3.4 million individuals worldwide.[3] Currently, the only treatment specific to A1ATD is intravenous administration of human plasma-derived native A1AT (nA1AT) at 60 mg kg^{-1} bodyweight once a week to restore A1AT to a normal circulating level.[4] However, nA1AT for augmentation therapy is expensive and its supply is insufficient for clinical demands.[5] Another drawback of nA1AT is its short half-life in vivo, which could be caused by instability, proteolysis, and oxidative deactivation. It has been reported that smokers with moderate A1ATD have an increased risk for the development of COPD (Chronic obstructive pulmonary disease).[6] It has further been demonstrated that the Met358 residue at the RCL of A1AT is susceptible to oxidation into methionine sulfoxide when exposed to tobacco smoke, resulting in activity loss (Figure 1a).[7] Moreover, the oxidized A1AT can be detected in patients with bronchiectasis and rheumatic diseases.[8] Therefore, protection of A1AT from oxidation is essential to A1AT function, and could significantly improve the therapeutic efficacy. While chemical conjugation such as PEGylation or fusion methods including Fc, HSA, PAS, XTEN, or in CDR loop can extend the circulation time of protein therapeutics, none of them can effectively protect the proteins from oxidative damage.[9]

Selenium is an essential trace element highly relevant to human health and multiple diseases.[10] Compared to sulfur, selenium has a larger atomic radius and has greater

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reactivity in many chemical reactions including nucleophilic substitution, redox reactions, and free radical reactions.[11] Because of their unique chemical reactivities and important roles in biology, selenium-containing polymers have been developed for drug delivery, self-adaptive materials, and enzyme mimics.[12]

We hypothesized that a selenium-containing polymer would be a promising conjugation "partner" for protein therapeutics such as A1AT to enhance their pharmacological properties. A bulky polymer could protect A1AT from proteolytic degradation, and more importantly, serve as a "sacrificial" agent to spare the oxidation of A1AT. Further, since the redox of selenium-containing moieties (e.g. selenomethionine) is often reversible under biologically relevant conditions towards completion of a full redox cycle,[13] the selenium moiety could be "re-charged" under reductive environment (Figure S1) and provide long-lasting protection against oxidative damages.

To test this hypothesis, we first expressed, purified, and characterized A1AT according to the published methods (Supporting Information and Figure S2, S3).[14] The purified protein showed potent inhibitory activity to NE (Figure 1b). Crystallographic studies have revealed that the binding of NE to A1AT cleaves its reactive loop, forming a covalent complex.[2] The reactive loop (residue Gly344 to Lys368) is critical for its activity (Figure 1a). Methionine 358 (depicted in orange), located at the A1AT active loop (colored in green) is prone to oxidation, resulting in protein deactivation (Figure 1a). *N*-chlorosuccinimide (NCS) was reported to selectively oxidize surface-exposed methionine residues to afford methionine sulfoxide.[15] Therefore, we tested the effect of oxidation of A1AT by incubating A1AT with 100 equivalents of NCS for 30 min at room temperature. The remaining inhibitory activity of A1AT was evaluated using an NE inhibitor screening kit. In this assay, the production of fluorescence from a specific fluorogenic substrate upon NE hydrolysis was detected and quantified. In the presence of inhibitors such as A1AT, the hydrolysis of the fluorogenic substrate was inhibited, which led to decreased fluorescent signals. As shown in Figure 1b, 10 nM A1AT can fully inhibit the activity of NE. Upon incubation with NCS, the NE activity was nearly all retained, suggesting the loss of inhibitory activity of A1AT by oxidation. As a control, the oxidation buffer containing equal amounts of NCS had little effect on NE activity.

Synthetic polypeptides and polypeptoids prepared by the ring-opening polymerization (ROP) of amino acid *N*-carboxyanhydride (NCA) have been widely used in biomedical engineering owing to its biodegradability and biocompatibility.[16] For example, polysarcosine (PSar) is an unstructured polypeptoid with excellent water solubility and non-fouling properties,[17] which has been proven as an outstanding alternative to PEG for protein conjugation.[18] We prepared two selenium-containing polypeptides (SePs) by means of copolymerization (SeP1 and SeP2, Figure 2a). Inspired by the high redox activity of selenomethionine (SeMet),[19] We introduced SeMet moieties to SeP1 through random copolymerization of sarcosine NCA (SarNCA) and selenomethionine NCA (SeMet NCA). Due to the hydrophobicity of SeMet, we found a Sar/SeMet molar ratio greater than 10:1 was necessary to make the polypeptide water-soluble. To increase the selenium content in the copolymer and improve the water solubility, we designed and generated the copolymer SeP2 to contain a water-soluble selenopolypeptide block P(EG_4-SeHC).[20] To ensure efficient conjugation, a flexible PSar block was extended from the N-terminus of the P(EG_4-SeHC). Both polymers have molecular weights ≈10 kDa as determined by SEC and contain a terminal maleimide for conjugation with a cysteine (Figure 2a and Figure S4).

To demonstrate the reactivity of the selenium-containing polymers against NCS, we mixed P(EG_4-SeHC) with 5 equivalents of NCS and NaOD in aqueous solution. In situ monitoring by ^{1}H NMR showed the clear shifting of the α-, β- and γ- hydrogen signals of P(EG_4-SeHC) (Figure 2b). The chemical shifts were similar to those of the selenoxide-containing polypeptide,[20] indicating that the polypeptide could be oxidized by NCS.

To determine whether the SePs could protect A1AT from oxidative deactivation, we mixed different equivalents of SePs with A1AT and 100 equivalents of NCS for 30 min at room temperature. The NE inhibitory activity was measured to evaluate the amount of unoxidized and active A1AT. As shown in Figure 2c and S5a, without the protection of the SePs, A1AT lost ≈80 % of activity under the oxidation condition. SeP1 did not exhibit significant protection to A1AT, while SeP2 could protect the activity of A1AT in a dose-dependent manner, presumably owing to the higher selenium content as compared to that of SeP1. When A1AT was mixed with 8 equivalents of SeP2, ≈50 % of the inhibitory activity was retained.

Since the SePs mixed with A1AT could counteract NCS oxidation, we reason that conjugation of SePs in proximity to Met358 in the active loop could provide antioxidation protection more efficiently. Based on the crystal structure of A1AT, Cys232 with a free thiol group is located on the surface and in proximity with Met358. Moreover, Cys232 is the only cysteine residue in the amino acid sequence of A1AT. Maleimide group can react specifically with sulfhydryl groups when the pH of the reaction mixture is between 6.5 and 7.5. Thus, we conjugated maleimide-containing selenopeptides with Cys232 of A1AT specifically by incubation of A1AT with 4–6 equivalents of SePs in reaction buffer of pH 7.0 at 4 °C overnight. The produced A1AT-SePs conjugates (A1AT-SeP1 and A1AT-SeP2) were purified by SEC to isolate the monomeric forms (Figure 2e) and characterized by SDS-PAGE (Figure 2f). It is worth noting that A1AT-SeP1 and A1AT-SeP2 migrated to molecular weights which seem larger than the theoretical ones on the SDS-PAGE. This is likely due to the large hydrodynamic volume of the polymer conjugated protein that caused retarded electrophoretic mobility on SDS-PAGE.[21]

Next, we measured the NE inhibitory activities of A1AT-SeP conjugates. 10 nM A1AT-SeP1 or A1AT-SeP2 was able to fully inhibit the activity of NE (Figure S5b) and exhibited a similar potency to that of A1AT (Figure 3a), demonstrating that SeP conjugation at Cys232 does not interfere with the activity of A1AT. To explore the influence

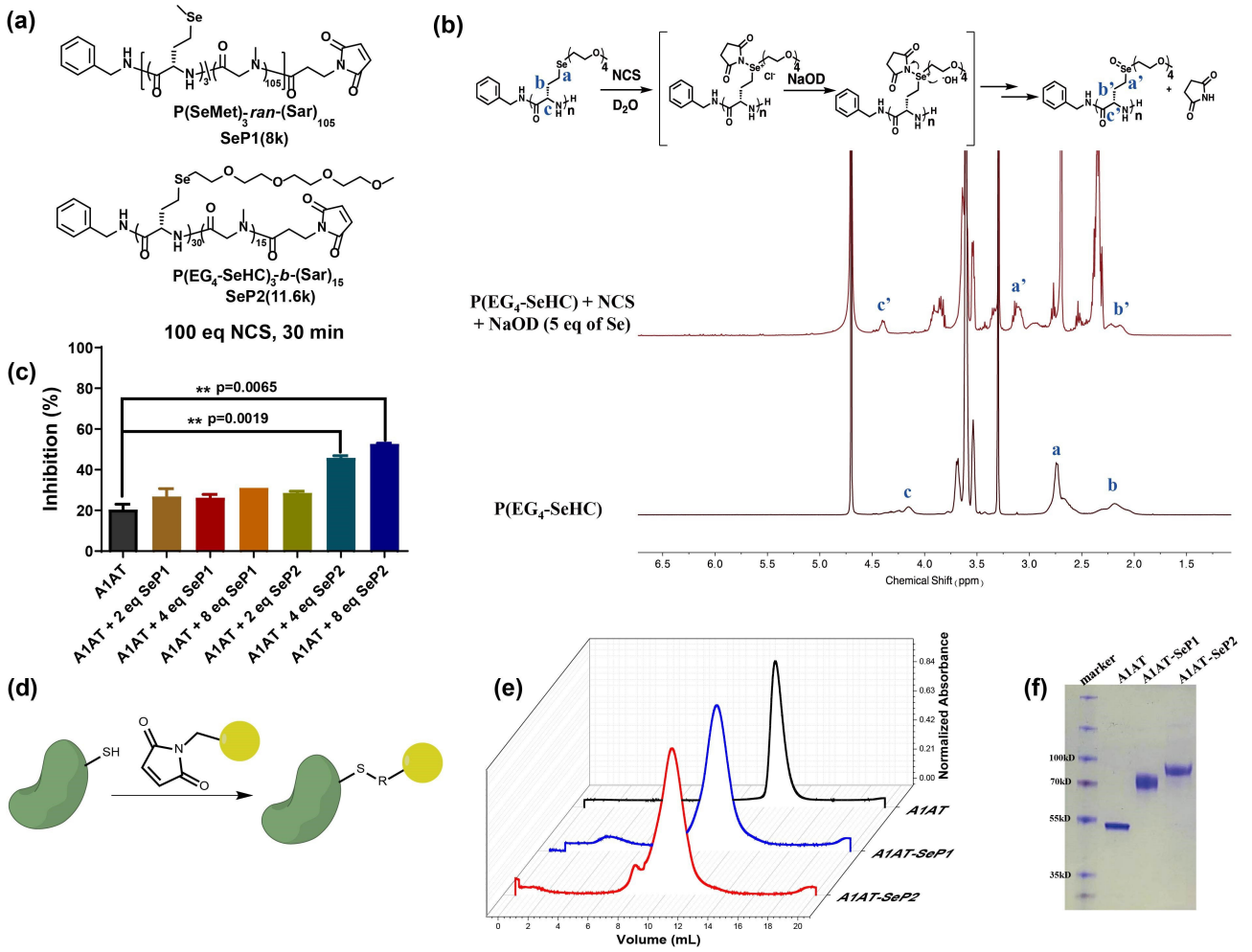

Figure 2. Structures and characterization of A1AT and polymer conjugate. a) Structure of the SePs. b) ^{1}H NMR spectroscopy of P(EG$_4$-SeHC) before and after NCS oxidation, c) NE inhibitory activity of 10 nM WT A1AT mixing with a series of equivalents of SePs after incubation with 100 equivalents of NCS for 30 min. d) Schematic picture of site-specific conjugation of A1AT (green) with SePs (yellow) via a cysteine-maleimide reaction. e) SEC of A1AT-SeP1, A1AT-SeP2 and A1AT (Superdex 75 Increase 10/300 GL). f) SDS-PAGE of purified A1AT, A1AT-SeP1, and A1AT-SeP2.

of NCS oxidation to the activities of A1AT and A1AT conjugates, we incubated 200 equivalents of NCS with A1AT, A1AT mixing with equivalent SePs, and A1AT-SeP conjugates for 30 min, respectively, and compared their remaining activities after oxidation. As expected, A1AT almost lost all of its inhibitory activity, while A1AT-SeP conjugates showed stronger antioxidant activity than A1AT mixed with equal equivalent SePs. In accordance with the antioxidant capabilities of the polymers, A1AT-SeP2 retained higher activity than A1AT-SeP1 under the same oxidation condition (Figure 3b, S5c).

Next, we incubated a series of concentrations of A1AT, A1AT-SeP1, and A1AT-SeP2 with 100 eq NCS to quantify and compare their remaining inhibitory activity. The inhibitory activity of A1AT was significantly decreased (IC50 = 22.0 ± 7.1 nM). In contrast, the activities of A1AT-SeP1 (IC50 = 7.7 ± 1.0 nM) and A1AT-SeP2 (IC50 = 5.7 ± 1.0 nM) were mostly retained (Figure 3c). When we increased NCS to 200 equivalents, A1AT-SeP2 (IC50 = 7.4 ± 1.4 nM) was clearly resilient to oxidation than A1AT-SeP1 (IC50 = 13.0 ± 1.8 nM) (Figure 3d). In addition, we investigated the oxidative deactivation in a time course. We first extended the incubation time from 30 min to 1 hour. Upon treatment of 200 equivalents of NCS, A1AT-SeP2 still maintained most of its activity (IC50 = 12.5 ± 2.5 nM), while a sharp activity decrease was observed for A1AT and A1AT-SeP1 (Figure 3e). These results suggested that higher selenium content of SeP2 could render more protection to A1AT than SeP1. Then we incubated A1AT and A1AT-SeP2 with 200 eq NCS for different amounts of time, A1AT was rapidly deactivated (Figure 3f), while A1AT-SeP2 was able to retain more than 50 % of its original activity at 40 min. Further, we decreased the amount of NCS from 200 equivalents to 40 equivalents and prolonged incubation time. A1AT-SeP2 retained about half of the original activity after 10 hours. Based on these results, we selected A1AT-SeP2 for further efficacy evaluation.

Next, a porcine pancreatic elastase (PPE) induced lung injury mice model was utilized to examine the protective efficacy of A1AT and A1AT-SeP2 in vivo. Lungs are the

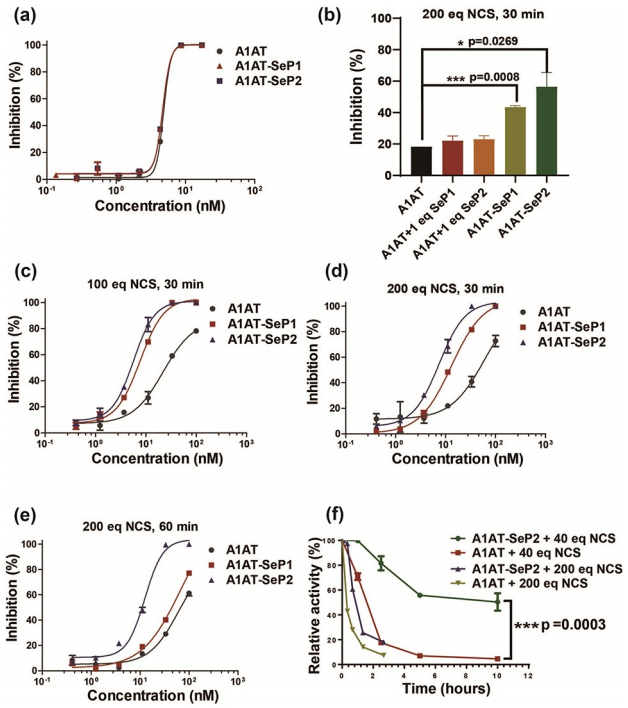

Figure 3. The antioxidant activities of selenopolypeptides. a) Inhibitory activities of A1AT, A1AT-SeP1 and A1AT-SeP2 against NE. b) NE inhibitory activity of 10 nM wt A1AT and A1AT conjugates after incubation with 200 eq NCS for 30 min. c) Inhibitory activities of A1AT, A1AT-SeP1, and A1AT-SeP2 after incubation with 100 eq NCS or 200 eq NCS for 30 min (d) or 1 hour (e) at r.t. f) Relative activity of A1AT and A1AT-SeP2 measured at different time points (20 min, 40 min, 80 min, 160 min, 1 h, 2.5 h, 5 h, 10 h) after incubation with 200 eq or 40 eq NCS at r.t. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

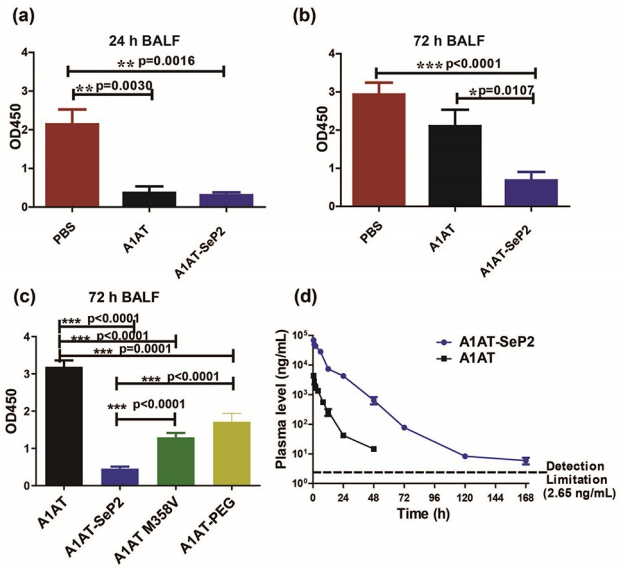

Figure 4. In vivo efficacy of A1AT and A1AT-SeP2. a–c) In vivo protection effect of A1AT, A1AT M358V, A1AT-PEG(10k) and A1AT-SeP2 in the PPE induced lung injury in mice. Mice were divided into three group and dosed with 50 μL PBS, 50 μL 130 μM A1AT, 50 μL 130 μM A1AT M358V, 50 μL 130 μM A1AT-PEG and 50 μL 130 μM A1AT-SeP2, respectively, at 24 h (a $n=6$) or 72 h (b $n=8$. c $n=8$) before the inhalable intratracheal instillation of PPE (150 U kg^{-1}). 24 h after PPE challenge, the animals were sacrificed for bronchoalveolar lavage and determination of MPO activity in the BALF. d) Pharmacokinetics of A1AT and A1AT-SeP2. Mice were divided into two groups ($n=6$) and received a single dose of 5 mg kg^{-1} via tail vein injection. Their blood samples were collected at various time points. Serum concentrations were analysed by ELISA. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

most oxygenated organ, leading to a highly oxidative environment on the surface of lung tissues. Therefore, drugs via pulmonary delivery are more susceptible to oxidation, making antioxidative capacity an important parameter for drug development and formulation via this dosing route. To establish an efficient, rapid and non-invasive lung administration method, test samples were administered by inhalable intratracheal instillation. Different doses of PPE were instilled to optimize appropriate dosage to induce lung injury. Myeloperoxidase (MPO) was selected as the pharmacodynamic biomarker because it is the most abundant proinflammatory enzyme stored in the azurophilic granules of neutrophilic granulocytes.[22] The inflammatory response in mice challenged with 100 μg PPE (150 U kg^{-1}) led to 15-fold increase of MPO activity in bronchoalveolar lavage fluid (BALF) (Figure S6). Mice treated with both A1AT and A1AT-SeP2 at 24 hours before the administration of PPE showed a significant reduction of MPO level compared to the vehicle group (Figure 4a). However, A1AT could not provide any significant protection when dosed at 72 hours before PPE administration. In contrast, dosing mice with A1AT-SeP2 at the same time point efficiently suppressed the inflammation by lung injury (Figure 4b), demonstrating its long lasting antioxidative capability in lungs. In addition, we compared the efficacy of A1AT-SeP2 with A1AT M358V mutant[23] and A1AT-PEG (10 kD) (Figure S7, S8). It was reported that the M358V mutant, whose oxidation-prone methionine was replaced with valine, minimized the liability of oxidative deactivation. A1AT M358V indeed exhibited stronger antioxidative ability than A1AT in vitro (Figure S9). However, when the NCS level was increased to 200 equivalents, A1AT M358V lost more activity than A1AT-SeP2, likely due to oxidation of other key residues (e.g. M351) in the active site.[24] Consistent with the in vitro results, both A1AT M358 V and A1AT-PEG showed stronger protective ability than A1AT, but were still lower than A1AT-SeP2, suggesting that the improvement in A1AT-SeP2 efficacy is from the additive effect of stealthy and antioxidant properties of SeP2.

PEGylation of a polypeptide reduces renal clearance and increases its half-life, and has been used to generate long-acting biotherapeutics. To evaluate whether SeP conjugation could render a similar effect, a single-dose pharmacokinetics study was performed using female C57BL mice via tail vein injection. The half-life of A1AT-SeP2 was markedly extended compared to A1AT, indicating the potential of A1AT-SeP2 as a long-acting therapy systemically (Figure 4d). Similar to PEG, the unit number and molecular weight of SeP2 can be rationally designed and generated by controlling polymerization conditions, which

can be utilized to modulate the circulating half-life and antioxidative capacity of the conjugated protein.

We also evaluated potential toxicity of SeP2. Mice were weekly administered with 1 mg kg^{-1} P(EG_4-SeHC) (2.7 fold higher than the dose used in efficacy studies) via intraperitoneal injection for 4 weeks. No obvious toxicities were observed and all mice in the polymer-dosing group exhibited similar body weights to the PBS control group, indicating the potential biosafety of SeP2 (Figure S10).

In summary, we designed and generated a novel class of selenopolypeptides which afforded strong antioxidant activity. We optimized the expression and purification of A1AT in *E.coli* and site-specifically conjugated SeP at A1AT Cys232 efficiently. Conjugation at the Cys232 of A1AT with SePs showed negligible influence to its NE inhibitory activity, while rendering strong protection against oxidants which are usually associated with inflammatory lung diseases.[25] We demonstrated that the SeP-A1AT conjugate showed strong and long-lasting antioxidant activities in vitro and in a mouse model. In addition, the conjugation of SePs significantly extended the circulation half-life of A1AT, which could facilitate the development of a long-acting therapy. In addition, P(EG_4-SeHC) showed a good safety profile in mice. Altogether this work sheds light on the potential of using antioxidant polymers as elements to enhance the pharmacological properties of easily oxidized biotherapeutics.[26]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: A1AT · Antioxidant Polymer · Protein Conjugation · Selenopolypeptide

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