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Site-Specific PEGylation of Human Thyroid Stimulating Hormone to Prolong Duration of Action

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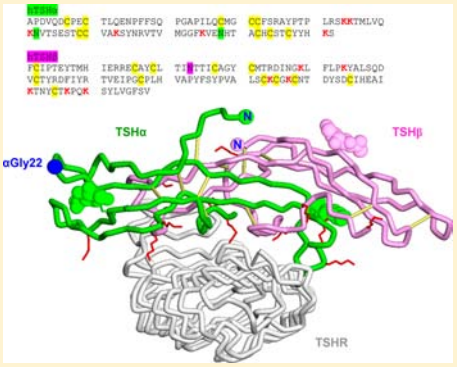
ABSTRACT: Recombinant human thyroid stimulating hormone (rhTSH or Thyrogen) has been approved for thyroid cancer diagnostics and treatment under a multidose regimen due to its short circulating half-life. To reduce dosing frequency, PEGylation strategies were explored to increase the duration of action of rhTSH. Lysine and N-terminal PEGylation resulted in heterogeneous product profiles with 40% or lower reaction yields of monoPEGylated products. Eleven cysteine mutants were designed based on a structure model of the TSH-TSH receptor (TSHR) complex to create unique conjugation sites on both α and β subunits for site-specific conjugation. Sequential screening of mutant expression level, oligomerization tendency, and conjugation efficiency resulted in the identification of the αG22C rhTSH mutant for stable expression and scale-up PEGylation. The introduced cysteine in the αG22C rhTSH mutant was partially blocked when isolated from conditioned media and could only be effectively PEGylated after mild reduction with cysteine. This produced a higher reaction yield, ~85%, for the monoPEGylated product. Although the mutation had no effect on receptor binding, PEGylation of αG22C rhTSH led to a PEG size-dependent decrease in receptor binding. Nevertheless, the 40 kDa PEG αG22C rhTSH showed a prolonged duration of action compared to rhTSH in a rat pharmacodynamics model. Reverse-phase HPLC and N-terminal sequencing experiments confirmed site-specific modification at the engineered Cys 22 position on the α-subunit. This work is another demonstration of successful PEGylation of a cysteine-knot protein by an engineered cysteine mutation.



INTRODUCTION

Thyroid stimulating hormone (TSH) regulates the endocrine function of the thyroid gland via its interaction with the G-protein coupled receptor TSHR, leading to the activation of secondary messenger pathways.[1] The stimulation of thyroid functions by TSH results in iodine uptake and organification, production and release of iodothyronine T3 and T4 from the gland, and promotion of thyroid growth. Recombinant human thyroid stimulating hormone (rhTSH or Thyrogen) is currently approved for thyroid cancer diagnostics and treatment.[2,3] Clearance of human and bovine-derived TSH has been reported with a half-life in monkeys of 35−100 min,[4,5] consistent with the relatively short circulating half-lives of growth hormones. rhTSH has about 2-fold improved half-life in rat compared to pituitary TSH due to higher sialylation of the oligosaccharides.[5] The short half-life makes a multidose regimen a necessity for clinical applications.

PEGylation of biotherapeutics has emerged as an effective drug modification strategy to improve therapeutic potential with less frequent dosing and greater compliance.[6,7] Although the conjugated products often display lower *in vitro* activity due to the presence of the bulky PEG group, the benefit from the increased pharmacokinetic profile can compensate for the activity loss and result in an improved pharmacodynamic profile.[8] PEGylation has been demonstrated in multiple cases to improve the circulating half-life of therapeutic proteins, including PEGylated Interferon-α,[9] G-CSF,[10] and anti-TNFα Fab[11] for the treatment of hepatitis C, neutropenia, and Crohn's disease, respectively.

PEGylation targeting lysine residues on a protein surface using N-hydroxysuccinimide (NHS) chemistry is well-established[7] as seen in FDA approved PegIntron and Pegasys. However, this approach often leads to heterogeneous product profiles due to the availability of multiple lysine residues on a protein. To reduce product heterogeneity and activity loss, site-specific modification has become a major focus for conjugation of biologics. Multiple approaches have been used in recent years, including selectively targeting the N-terminus of a protein,[12] engineered Cys residues for thiol conjugation (Cimzia), engineered unnatural amino acids with keto chemistry,[13] and conjugation at oxidized carbohydrates with hydrazide and aminoxy chemistries.[14,15] Site-directed Cys introduction has many advantages due to the high efficiency of the maleimide reaction and the availability of a large selection of maleimide PEG derivatives. However, the newly introduced Cys may not be completely available for conjugation due to cysteinylation and glutathionylation.[16] Dimerization, oxidation, and aggregation have also been observed via

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unpaired Cys residues on a protein,[17] and thus may make it challenging as an engineered site for PEGylation.

rhTSH is a noncovalently linked heterodimeric glycoprotein (Figure 1). The 92-amino-acid α-subunit (TSHα) has the

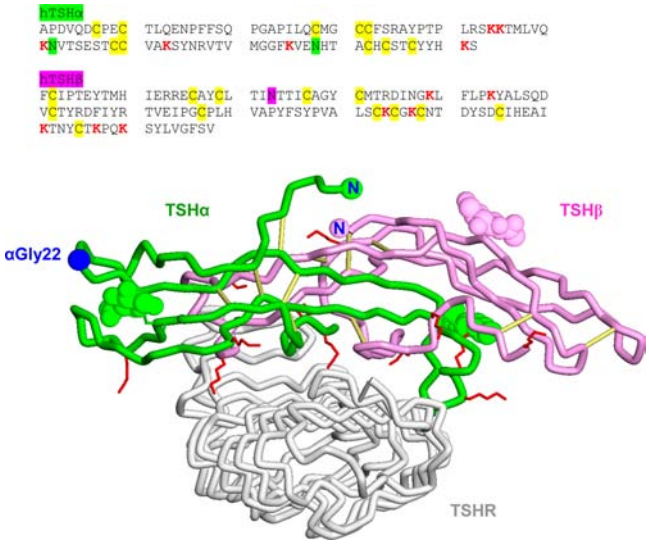

Figure 1. rhTSH amino acid sequences and TSH-TSHR structural model with important amino acids highlighted. The complex of noncovalently linked TSH heterodimer with TSH receptor is shown. The lysines in the sequence are in red. Cysteines are in yellow. The three glycosylation sites are colored with their backbone colors: green for α-subunit and purple for β-subunit. The TSHR is displayed in silver. The Cα of αGly22 is highlighted and labeled in blue.

identical amino acid sequence as that of human chorionic gonadotropin (hCG), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). The 118-amino-acid β-subunit (TSHβ) is unique to TSH, and determines its receptor specificity. TSH is a member of the cysteine-knot growth factor superfamily, with 22 out of the total 210 amino acids being cysteine residues (Figure 1). Both subunits are cysteine-rich, and all cysteines are disulfide-bonded based on the published crystal structures of family members hCG and hFSH.[18] There are three glycosylation sites on TSH, two on the α-subunit (Asn52 and Asn78) and one on the β-subunit (Asn23). The structure of the TSH-TSHR complex is not available to date, but structural models built from the published FSH-FSHR structure have been used to understand the structure/function of the TSH-TSHR interaction.[19] Analysis of interactions in the FSH-FSHR and TSH-TSHR complexes suggests that the α-chains of both hormones are involved in the receptor activation process while the β-chains are involved in defining binding specificity.[19] The components important for TSH expression and activity have been found to be a "composite binding domain", consisting of α-helix (α40−46), αLys51, αAsn52-linked oligosaccharide, the C-terminus of the α-chain, the so-called keutman's loop (β31−52), and the "seat-belt" β88−105 in the β-subunit,[1] which make extensive contacts with the TSHR.

In the current work, we evaluated lysine, N-terminal, and engineered cysteine PEGylation strategies to enhance the duration of action of rhTSH.

EXPERIMENTAL PROCEDURES

Materials. rhTSH and anti-rhTSH monoclonal detection antibody TS8 were produced by Genzyme Corp. *N*-Hydroxysuccinimide (NHS)-PEG, maleimide (Mal)-PEG, and aldehyde-PEG reagents were purchased from NOF America Corporation (White Plains, NY). The TSH receptor auto-antibody second-generation ELISA kit from RSR Limited (U.K.) was purchased through Kronus Inc. (Star, ID). Other chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise stated.

Lysine Conjugation. NHS (*N*-hydroxysuccinimide)-PEG (50 mg/mL in dH_2O) was added to a final concentration of 0.8 mg/mL rhTSH at varying (PEG:protein) ratios (e.g., (0.5:1), (1:1), (2:1), (4:1), (8:1)) in PBS (phosphate-buffered saline) buffer, pH 7.2. Incubation was done for 1.5 h or overnight at 25 °C. Size exclusion chromatography (SEC) fractions of mono-PEGylated species were collected and submitted for *in vitro* TSH receptor binding analysis.

N-Terminal Conjugation. Aldehyde PEG stock (100 mg/mL in reaction buffer) was added to a final concentration of 5 mg/mL rhTSH at varying (PEG:protein) ratios in 100 mM sodium acetate, pH 5.6, with 20 mM sodium cyanoborohydride. Incubation was carried out for 16 h at 25 °C or for up to 2 days at 8 °C. The reaction was quenched with 0.1 volume of 1 M tris, pH 7.5, for 3 h at 25 °C. The reaction mixture was analyzed by SDS-PAGE with Coomassie blue and PEG stain to monitor the total yield and the percent mono-PEGylated species. The mono-PEGylated rhTSH was purified by size exclusion chromatography (SEC-HPLC).

Generation of TSH Cys Mutants. DNAs encoding rhTSH genes (including its signal peptides) were synthesized and cloned into a Gateway entry vector pDONOR221. A DNA sequence encoding a 12-amino-acid HPC4 peptide tag was added after the C-terminal of the TSH β-subunit for enhanced detection and purification by an antiprotein C antibody clone HPC4.[20] Oligonucleotide-based site-directed mutagenesis was used to introduce Cys mutations at multiple sites on both rhTSH subunits by the Quikchange kit (Stratagene, CA). The resulting wild-type and mutant vectors were shuffled into expression vectors pCEP4.DEST via Gateway cloning. Proteins were prepared from transiently transfected HEK293 cell media for biochemical and cell-based assays. For expression level assessment, 4.5 mL of conditioned media was incubated with anti-HPC4 beads (Roche) in the presence of 5 mM calcium chloride ($CaCl_2$). Medium-scale protein purification was performed with an affinity column prepared with the anti-HPC4 beads on an ÄKTA FPLC. Larger scale Cys mutants were prepared from CHO pools. DNA encoding the wild-type (WT) and αG22C mutant rhTSH genes were codon-optimized and synthesized, and then cloned into CHO expression vectors pGEN600 and pGEN620 for transient transfection in CHO cells. Transfected CHO cells were amplified with methotrexate (MTX) selection. The resulting CHO pools were used for scale-up protein production.

Site-Specific Cys PEGylation. For small-scale PEGylation of transiently produced Cys mutants, TCEP-agarose beads (Pierce Biotechnology, Inc.) were used to prepare the protein before conjugation. Briefly, TCEP-beads were washed several times with conjugation buffer (25 mM sodium phosphate, 25 mM sodium chloride, 2 mM EDTA, pH 7.0) and mixed 1:1 (v/v) with rhTSH mutants for 15 min with rotation. The reduced protein was separated from the beads by centrifugation and

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incubated for 2 h at 25 °C allowing proper refolding. A 5:1 molar ratio of malPEG was added to the protein and the mixture was incubated for 2 h at 25 °C. The PEGylation reaction was quenched with a 2:1 molar ratio of freshly prepared cysteine. The mono-PEGylated TSH mutants were analyzed by SDS-PAGE and purified by SEC-HPLC. For large-scale PEGylation of stably produced αG22C rhTSH, cysteine (2 mM), or TCEP (various ratios; see Results) was added to the mutant (1−2 mg/mL) and incubated overnight (for cysteine) at 4 °C or for one hour (for TCEP) at 25 °C to remove the cap on Cys22. The mixture was diafiltered into PEGylation buffer (10 mM sodium phosphate, 2 mM EDTA, pH 7.0). MalPEG was added to the protein to attain 5× molar excess and incubated for 2 h at 25 °C. The PEGylation was stopped with 2× cysteine and the yield was checked by SEC-HPLC. The pH of the reaction mixture was lowered to pH 5.0 and then loaded on a Mono S column for purification.

SDS-PAGE Analysis and PEG Staining. A NuPAGE Novex 4−12% bis-tris gel (Life Technologies, NY) was loaded with 4−5 μg of PEGylation reaction mixture per lane and electrophoresis was performed with MOPS running buffer (Life Technologies, NY). The apparatus was placed in an ice bath to keep the gel cold during electrophoresis. PEG staining was done according to a modified procedure of Kurfürst.[9,21] Briefly, the gel was rinsed with distilled water, followed by 5% (w/v) barium chloride for 10 min, and then rinsed again with distilled water before staining with 10-fold dilution of 0.1 N iodine solution (EMD Chemicals, NJ). Once the PEGylated species were visible, the background staining was rinsed off with distilled water and the gel was scanned immediately. PEG stain was then removed with destaining solution (10% acetic acid, 20% methanol) prior to Coomassie blue staining.

SEC-HPLC Analysis of PEGylated rhTSH. The reaction mixture of PEGylated rhTSH was analyzed on a Superdex 200 10/300 GL column (13 μm, 10 × 300 mm, GE Healthcare, NJ) at 280 nm using an Agilent 1100 HPLC system with a diode-array UV detector (Agilent Technologies, CA). Sample was eluted isocratically with a flow rate of 0.4 or 0.5 mL/min with 150 mM sodium chloride, 50 mM sodium phosphate, pH 7.0, for 60 min.

Purification of Large-Scale PEGylated rhTSH. The reaction mixture of PEGylated rhTSH was purified over a Mono S 10/100 GL column (10 μm, 10 × 100 mm, 8 mL bed volume, GE Healthcare, NJ) using an ÄKTA Purifier (GE Healthcare, NJ) at 280 nm and a flow rate of 4 mL/min. After equilibrating for 10 column volumes (cv) at 100% A (10 mM sodium acetate, pH 5.0), the elution gradient increased from 0% to 50% B (10 mM sodium acetate, 1 M sodium chloride, pH 5.0) over 25 cv, followed by a column wash at 100% B for 5 cv and equilibration at 100% A for 5 cv.

***In Vitro* TSH Receptor Binding Assay.** rhTSH was biotinylated with 1.7 to 1.8 biotins per protein using the Chromalink Biotin Labeling Kit according to the manufacturer's protocol (SoluLink, CA) and buffer-exchanged into 150 mM sodium chloride, 50 mM sodium phosphate, pH 7.0, with a Zeba spin column (Thermo Scientific, IL). *In vitro* measurement of receptor binding was performed by competition of biotinylated rhTSH and purified PEG-rhTSH conjugates for binding to porcine TSH receptor immobilized onto 96-well plates, supplied in the TSH Receptor Auto-antibody second-generation ELISA kit from RSR Limited (U.K.). PEG-rhTSH conjugates were serially diluted 1:5 from 16 μM to 41 pM in assay buffer (100 mM HEPES pH 7.5, 20 mM EDTA, 1% BSA, 0.5% Triton X-100) and mixed 1:1 with 0.7 μg/mL biotinylated rhTSH in assay buffer. The mixture was added to each receptor-coated well and incubated for 25 min at 25 °C before washing away unbound biotinylated or PEGylated rhTSH. Streptavidin peroxidase was added for 20 min at 25 °C according to the RSR Limited ELISA protocol to determine the amount of biotinylated rhTSH bound to the plate. The plate was then washed three times to remove excess unbound streptavidin peroxidase, and then tetramethylbenzidine (TMB) was added to each well and incubated in the dark for 30 min at 25 °C. The reaction was quenched with 0.5 M sulfuric acid stop buffer, and the absorbance of each well was read at 450 nm using a SpectraMax 340pc plate reader (Molecular Devices, CA). The data were fit using a sigmoidal dose response equation with GraphPad *Prism* software to generate IC_{50} values.

Animal Studies. The pharmacodynamics of PEGylated Cys mutant TSH (40 kDa PEG αG22C rhTSH) was evaluated and compared to the pharmacodynamics of rhTSH in T3 pellet implanted Sprague−Dawley rats. Animals were anesthetized with isoflurane and a 1.5 mg T3 pellet (T-261, Innovative Research of America) was implanted subcutaneously using a trochar. At three days post pellet implantation, a single dose of vehicle, rhTSH, or PEGylated Cys mutant TSH (40 kDa PEG αG22C rhTSH) was administered intramuscularly to male and female jugular vein cannulated rats at a dose of 0.4 mg/kg. Due to dose volume limitations, animals received test articles in the form of two intramuscular injections into the quadriceps muscle. Legs were alternated for dosing. Blood samples were collected from the animals predose and at the following postdosage time points: 6, 24, 48, 72, 96, and 168 h. Blood was collected from the single port jugular cannula. Approximately 250 μL of whole blood was collected into serum separator tubes and the blood was allowed to clot for a minimum of 30 min. Tubes were spun in a centrifuge at 10 000 rpm for 5 min. Serum T4 concentration was measured by ACE clinical chemistry system (Alfa Wassermann Diagnostic Technologies, LLC) according to the manufacturer's protocol.

Determination of PEGylation Site by Peptide Mapping and N-Terminal Sequencing. 40 kDa PEG αG22C rhTSH and rhTSH control (20 μg each) were reduced in 6 M guanidine hydrochloride, 38 mM dithiothreitol, 0.1 M Tris, pH 8.5 overnight at 25 °C. The samples were then alkylated by adding iodoacetamide to a final concentration of 49 mM and incubating overnight at 25 °C. The reduced and alkylated samples were dialyzed into 0.1 M Tris, pH 8.5 using 3500 kDa cutoff Slide-A-Lyzer cassettes (ThermoFisher Scientific) before digesting with 1/25 (E/S) trypsin overnight at 37 °C The digest reactions were quenched by adding trifluoroacetic acid to a final concentration of ∼0.07%. Digests were loaded onto a Poroshell 300SB-C8 column (2.1 × 75 mm, 5 μm particles, Agilent Technologies, CA) that was held at 50 °C and pre-equilibrated with 98% solvent A (0.1% aqueous trifluoroacetic acid) and 2% solvent B (0.08% trifluoroacetic acid in acetonitrile). The column was eluted with a linear gradient of 2% to 60% B in 25 min at 0.4 mL/min. The fragment peak eluting at ∼25 min was collected and analyzed with the preprogrammed pulsed liquid method on a Procise protein sequencer (Applied Biosystems).

RESULTS

Lysine and N-Terminal PEGylation. Lysine conjugation with 40 kDa *N*-hydroxysuccinimide ester (NHS) PEG was first evaluated. This approach generated a heterogeneous population

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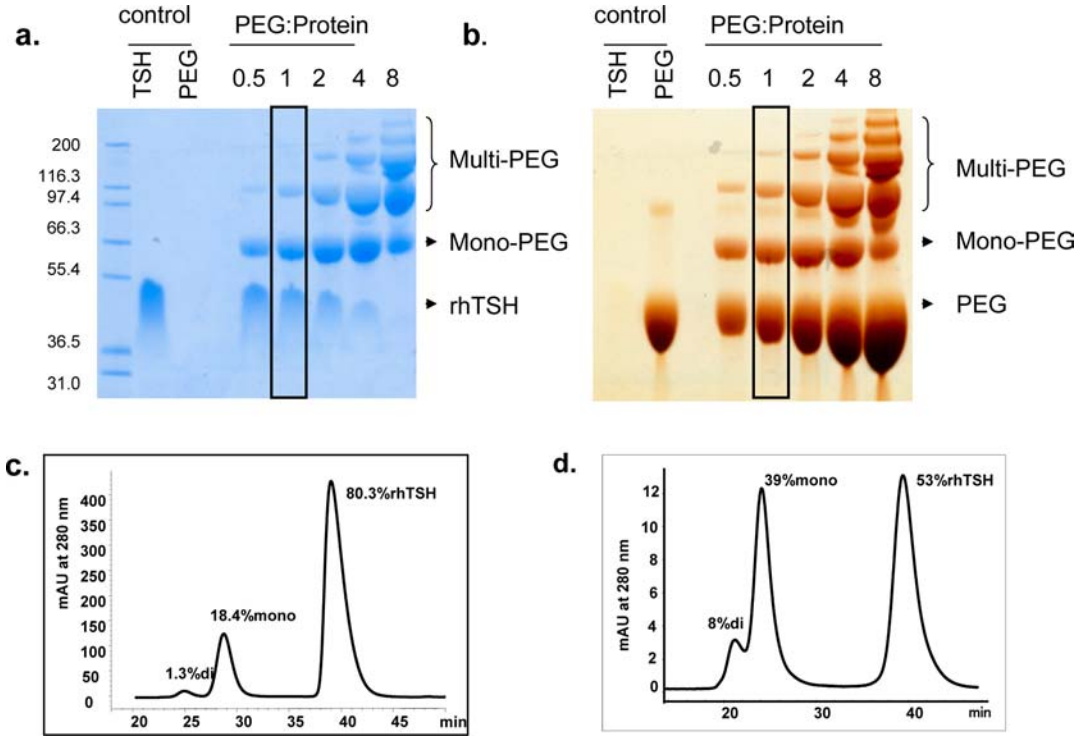

Figure 2. Lysine- and N-terminal PEGylation reactions. SDS-PAGE analysis of PEGylation reaction mixture of lysine PEGylation with different PEG:protein ratio are shown in part a (Coomassie blue stain) and part b (PEG stain). Reaction mixture with 1:1 PEG:protein ratio is highlighted with boxes, which was further analyzed on a SEC-HPLC (c). Part d is a SEC-HPLC profile of a representative N-terminal PEGylation reaction.

of mono- and multi-PEGylated species due to the presence of multiple Lys residues (Figure 2). Increasing the NHS-PEG in the reaction did lead to complete PEGylation of rhTSH, however, the products were mostly multi-PEGylated species as seen on the SDS-PAGE gel. Lower PEG ratios (0.5× to 1× molar) in the reaction led to lower overall PEGylation yield but higher mono-PEGylation and easier separation from the multi-PEG species. SEC-HPLC analysis of the reaction mixture generated by 1:1 PEG:Protein ratio (Figure 2c) results in a low mono-PEGylation yield (18%). The mono-PEGylated species were collected from SEC fractions and subjected to an *in vitro* TSH receptor binding assay. The results showed that the mono-40 kDa Lys-PEG-rhTSH had a 36.4-fold lower affinity than the control TSH (Table 1).

Table 1. IC_{50} Values from Competitive ELISA Binding Assay

sample	IC_{50} (nM)	fold difference (relative to rhTSH)	fold difference (relative to αG22C)
rhTSH	25 ± 6	1	
40 kDa Lysine PEG	911 ± 625	36.4	
40 kDa N-terminal PEG	366 ± 167	14.6	
αG22C	42 ± 9	1.7	1
40 kDa αG22C linear PEG	193 ± 67		4.6
40 kDa αG22C GL-2 PEG	567 ± 87		13.5
40 kDa αG22C GL-4 PEG	480 ± 30		11.4
50 kDa αG22C GL-3 PEG	226 ± 21		5.4
60 kDa αG22C GL-2 PEG	668 ± 213		15.9

N-terminal PEGylation with a 40 kDa aldehyde PEG was then tested. Low pH is known to increase the PEGylation specificity on the N-terminus of a protein over lysines.[10] However, we found that lower pH destabilizes the α-β dimer structure of rhTSH. pH 5.6 was chosen as the final PEGylation condition after pH titration studies. The mono-PEGylation yield was found to be 39% at 2:1 PEG:protein ratio (Figure 2d), comparable to the lysine conjugation at similarly high PEG:protein ratios. The mono-PEGylated rhTSH was purified by SEC-HPLC and analyzed for receptor binding and N-terminal sequencing. Since PEGylation of rhTSH at the N-terminus inhibits Edman chemistry and results in "blocked" N-termini, the site-specific mono-PEGylation yield was measured by quantifying the percent of blocked N-termini in N-terminal amino acid sequencing. The results indicated that 36% of the α-subunit and 49% of the β-subunit were PEGylated at the N-terminus. The sum of N-terminal PEGylation determined from blocked N-termini was 85%, suggesting ~15% nonspecific PEGylation on other sites. This N-terminal 40 kDa mono-PEGylated rhTSH also resulted in reduced activity (Table 1), about 14.6-fold lower than that of the wild-type rhTSH control.

Design and Screening of Single Cys Mutants. TSH mutants were designed and prepared to introduce a single unpaired cysteine on various parts of both TSH subunits for site-specific PEGylation. These mutants were designed for efficient conjugation while minimizing the effect of PEG on protein folding and receptor binding. Several considerations were applied when designing the sites for the cysteine mutation. First, sites were avoided at or adjacent to the interface with the receptor, the α/β subunit dimerization interface, and existing cysteines based on our structural model of the TSH-TSHR complex (Figure 1). Second, we avoided sites that, when mutated, result in dramatic loss in specific activity based on

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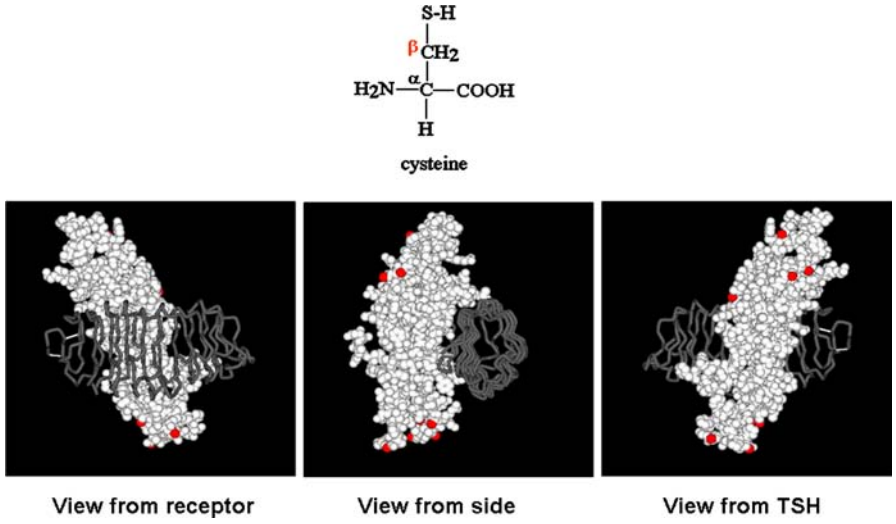

Figure 3. Multiview space-filling models of Cys mutants designed in this work. A dozen Cys mutants were designed based on a structural model and prior literature. A space-filling model is shown with the β-carbon of the target mutant side chain shown in red, which indicates that it is solvent-exposed. TSH is highlighted in white, TSH receptor in gray.

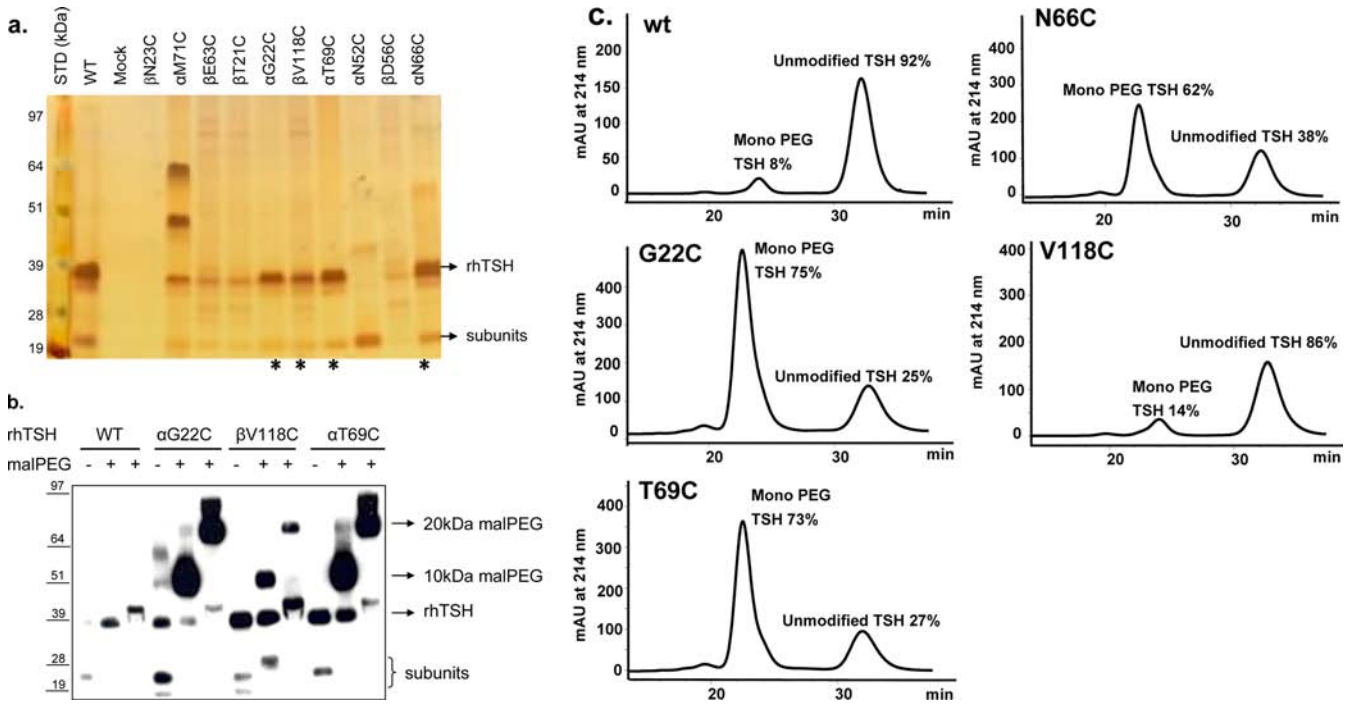

Figure 4. Production and characterization of rhTSH Cys mutants. The batch-purified mutants were analyzed on a silver-stained nonreducing SDS-PAGE to assess expression level and oligomerization of the mutants (a). Selected mutants were tested with PEGylation and analyzed by Western blot (b) and SEC-HPLC (c). αG22C was chosen as the lead for further production in CHO cells.

previous reports.[22,23] Finally, we favored sites that are fully solvent exposed for subsequent PEGylation and are evenly spaced to cover most of the TSH surface on the opposite side of the receptor binding site to fully evaluate PEGylation feasibility at each region. The β-carbon positions of the selected mutants were exposed to solvent in our structure model as shown in Figure 3. This predicted that these positions were likely to be accessible to PEGylation reagents when mutated to cysteines. The αN52C, αN78C, and βN23C were designed to create a conjugation site to replace the glycosylation.

The HPC4-tagged TSH mutant DNA in pCEP4 vector was used for transient expression in HEK293 cells. Conditioned media from 4.5 mL transfections were collected and incubated with HPC4-agarose beads for quick batch purification. The protein was eluted from the beads and analyzed by nonreducing SDS-PAGE followed by silver stain to assess the expression of the mutants (Figure 4a). Wild-type rhTSH had the highest expression level. Mutants αN66C, αG22C, αT69C, and βV118C had the best expression levels (see lanes with * symbol) among the 11 mutants. The αM71C mutant had a significant level of expression but formed higher molecular weight bands, presumably due to oligomerization via the introduced cysteine. The three mutants at the glycosylation sites αN52C, αN78C (not shown), and βN23C showed very

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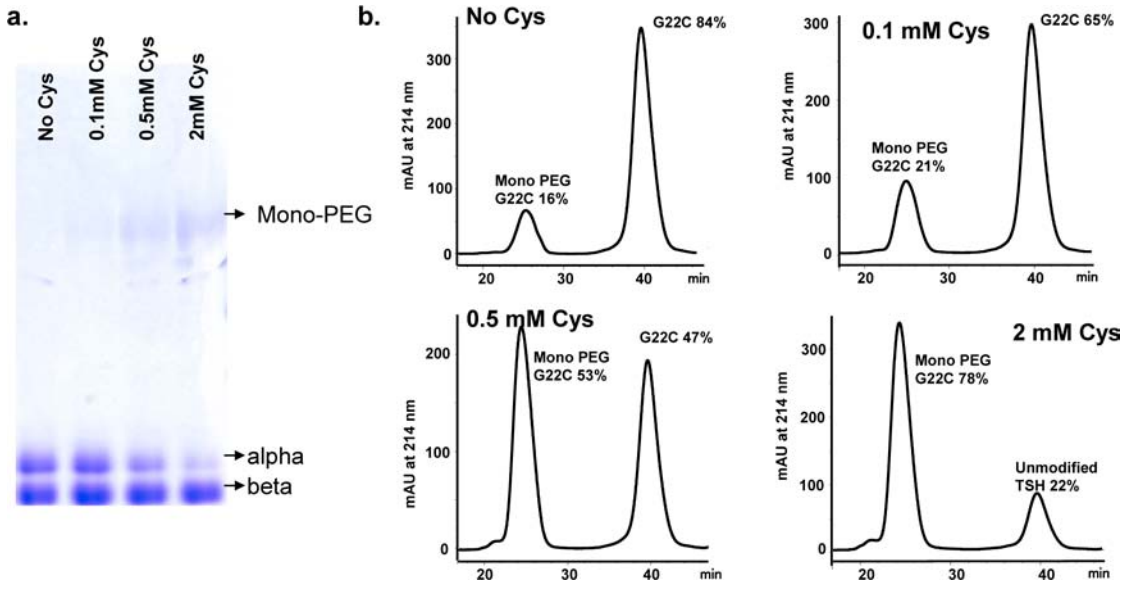

Figure 5. Optimization of αG22C TSH PEGylation conditions. Various conditions were tested to maximize the yield of mono-PEGylation, with minimum introduction of multi-PEGylated species. PEGylation reactions after reduction by various concentrations of cysteine were analyzed on SDS-PAGE (a) and SEC-HPLC (b). The most effective PEGylation was achieved with 2 mM cysteine reduction at pH 7.0.

low or no expression, suggesting the importance of the carbohydrates to protein folding or expression. Bands at the bottom of the gel may represent the dissociated β-subunit that co-purified with the anti-HPC4 affinity beads, as two subunits dissociate to some degree under gel electrophoresis conditions. The αN52C mutant appears to impact the stability of the α-subunit or its interaction with the β-subunit since only the β-subunit was detected on the gel.

Mutants with the highest expression and the expected migration on SDS-PAGE were isolated from 360 mL conditioned media of a HEK293 transfection for further characterization. The conditioned media was concentrated and the mutants were purified on an anti-HPC4 affinity column. The purified TSH were preincubated with TCEP beads and then tested for their reactivity toward 10 kDa and 20 kDa malPEGs. Anti-HPC4 Western blot (Figure 4b) and SEC-HPLC profiles (Figure 4c) indicated that αG22C and αT69C were most effectively PEGylated, followed by αN66C, βV118C, and αN66C. The electrophoresis conditions again resulted in dissociated subunits at the bottom of the gel. The αG22C mutant was chosen as the lead for stable expression in CHO cells and further characterization since it displayed the highest PEGylation efficiency.

αG22C rhTSH PEGylation. αG22C rhTSH was expressed and purified from CHO cells. The protein was purified predominately as a monomer as measured by SEC-HPLC (data not shown). There was a small amount, ~8% of higher molecular weight species (HMWS), presumably due to dimerization through the free unpaired cysteine at this position. The HMWS were removed using a SEC column on an ÄKTA before the PEG reaction. When the CHO expressed αG22C rhTSH was directly used for PEGylation, the yield was only about 15−20% (data not shown). This is likely due to the modification on Cys22 that has reduced its reactivity toward malPEG. It has been reported that a free cysteine on recombinant proteins can be cysteinylated or glutathionylated when expressed from CHO cells.[24,25] This would produce an unpaired cysteine that is "capped" with a mixed disulfide bond and unavailable for modification. PEGylation was found to improve significantly if the αG22C rhTSH protein was reduced first with DTT or TCEP to remove the cap. However, high levels of di-PEGylated species were detected by SDS-PAGE and SEC, despite multiple reductant titrations and time course experiments (data not shown). The appearance of higher PEGylated species suggests the reduction of the existing disulfide bonds. Cysteine, a mild reducing agent, was found to be better at removing the αG22C cap for optimal PEGylation while limiting the levels of multi-PEGylated species (Figure 5). The 2 mM cysteine reduction condition was used to generate various preparations of PEGylated αG22C for *in vitro* and *in vivo* characterization. The SDS-PAGE reaction profile (Figure 5a) indicates that the α-subunit is PEGylated, as seen from the decreased band intensity that corresponds to the α-subunit. This is consistent with the cysteine mutation introduced on the α-subunit.

Effect of PEGylation on TSH Receptor Binding. A competitive ELISA binding assay was developed to monitor the effect of PEGylation on TSH receptor binding. An IC_{50} value (concentration to compete half of the biotin TSH binding to porcine TSHR) was used for quantitative comparisons. The results for WT, αG22C rhTSH, and 40 kDa linear PEGαG22C rhTSH are shown in Figure 6. The results suggest that substitution of Gly22 with a Cys has minimum effect on rhTSH receptor binding. The IC_{50} calculation indicates a modest 1.7-fold change when compared to WT rhTSH (Table 1). Interestingly, conjugation of 40 kDa linear PEG αG22C rhTSH at this engineered Cys has a meaningful effect on receptor binding. The decrease is about 4.6-fold (Table 1) in the IC_{50} value for the 40 kDa linear PEGαG22C rhTSH compared to that of the unconjugated αG22C rhTSH.

MalPEG of different sizes (40−60 kDa) and branches were used to make a variety of αG22C PEG species. GL-2 represents PEGs with two branches that diverge near the conjugation site. GL-3 is a "Y"-shaped PEG, linear proximal to the conjugation site and then branching out to become two arms in the middle of the poly(ethylene glycol) chain. GL-4 is designated for PEGs of 4 branches close to the conjugation site. These PEG variants were conjugated to αG22C rhTSH, followed by purification of

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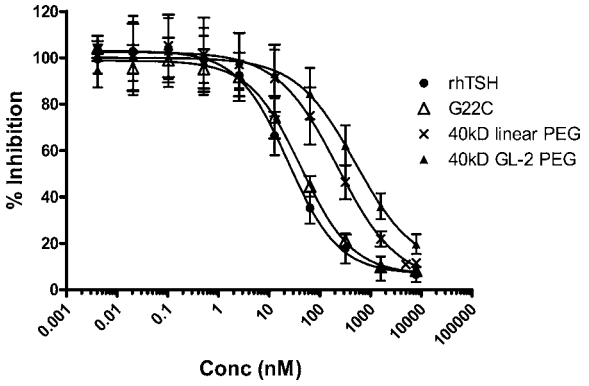

Figure 6. Competitive TSH receptor binding by rhTSH, αG22C rhTSH, and 40 kDa αG22C PEG-rhTSH.

the monoconjugated species and tested in the receptor binding assay. The additional PEG mass or branching appears to have a modest effect on receptor binding, within 3-fold of the 40 kDa linear PEG αG22C rhTSH, with the exception of the 60 kDa PEG αG22C rhTSH (Table 1).

Rat Pharmacodynamics Test. It has been previously shown that the *in vivo* clearance rate of a branched 40 kDa PEG-Fab was reduced 2-fold relative to the linear 40 kDa PEG-Fab.[26] Therefore, we decided to choose the 40 kDa branched monoPEGylated αG22C rhTSH for a pharmacodynamic study in rats. Sprague–Dawley rats were implanted with slow-release T3 pellets before rhTSH administration to suppress endogenous T4 during the study period so that the amount of T4 released in the postdose serum was solely from the stimulation by rhTSH or PEG-rhTSH conjugates. The result suggests that 40 kDa PEG αG22C rhTSH was biologically active even though its *in vitro* binding affinity was reduced (Figure 7). Moreover, the T4 level was significantly higher in the serum of 40 kDa PEG αG22C rhTSH treated rats at the later time points (48 and 72 h postdosing). Thus, the 40 kDa PEG αG22C rhTSH has an extended duration of action compared to rhTSH in this rat model.

Confirmation of Conjugation Site for PEGylated αG22C rhTSH. The purified 40 kDa PEG αG22C rhTSH was further tested to confirm that the PEG group was conjugated selectively to the G22C site on α-subunit of rhTSH. The reducing SDS-PAGE result showed that PEGylation was selective for the α-subunit as determined by Coomassie blue and PEG stains (Figure 8a). There was no visible unPEGylated α-subunit seen on the gel, suggesting complete subunit-specific PEGylation. Comparative peptide mapping for rhTSH and 40 kDa PEG G22C TSH was performed. Trypsin digests of the two proteins at a 1:25 enzyme:protein ratio were performed overnight at 37 °C. The reverse-phase HPLC (RP-HPLC) map of the 40 kDa PEG αG22C rhTSH sample indicated the depletion of the AT1 fragment (the first tryptic fragment of the α-subunit) found in rhTSH, which shifted to a longer elution time and a broader peak due to PEGylation (Figure 8b). The residual AT1 peak found in the PEGylated G22C sample may represent the loss of PEG due to overnight incubation at elevated temperature and the RP-HPLC running condition. The AT1 fragment contains five Cys residues in addition to the introduced residue at position 22. To further refine the site of conjugation, the hydrophobic RP-HPLC peak attributed to PEGylated tryptic

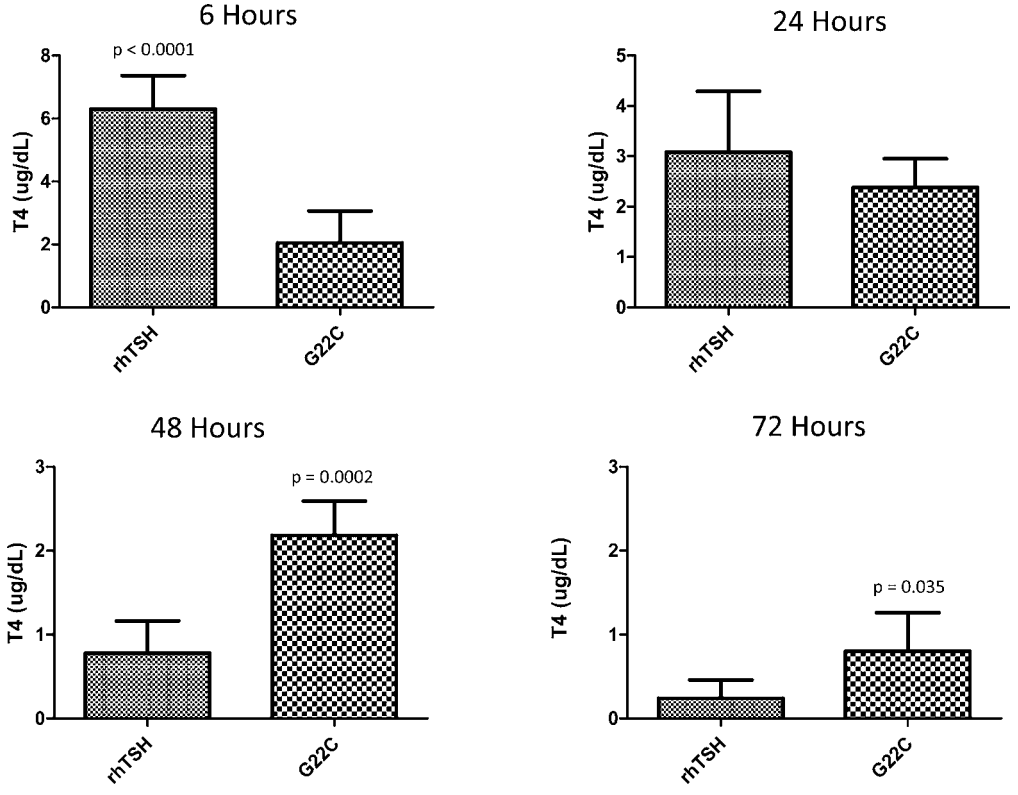

Figure 7. Rat PK/PD results. Thyroid hormone T4 level in circulation was monitored after administration of rhTSH control and 40 kDa αG22C PEGrhTSH. T4 levels at 6, 24, 48, and 72 h are shown. The difference was found to be statistically significant (p value displayed above bars). The result suggests that 40 kDa-αG22C PEG rhTSH is biologically active and has a longer duration of action than rhTSH.

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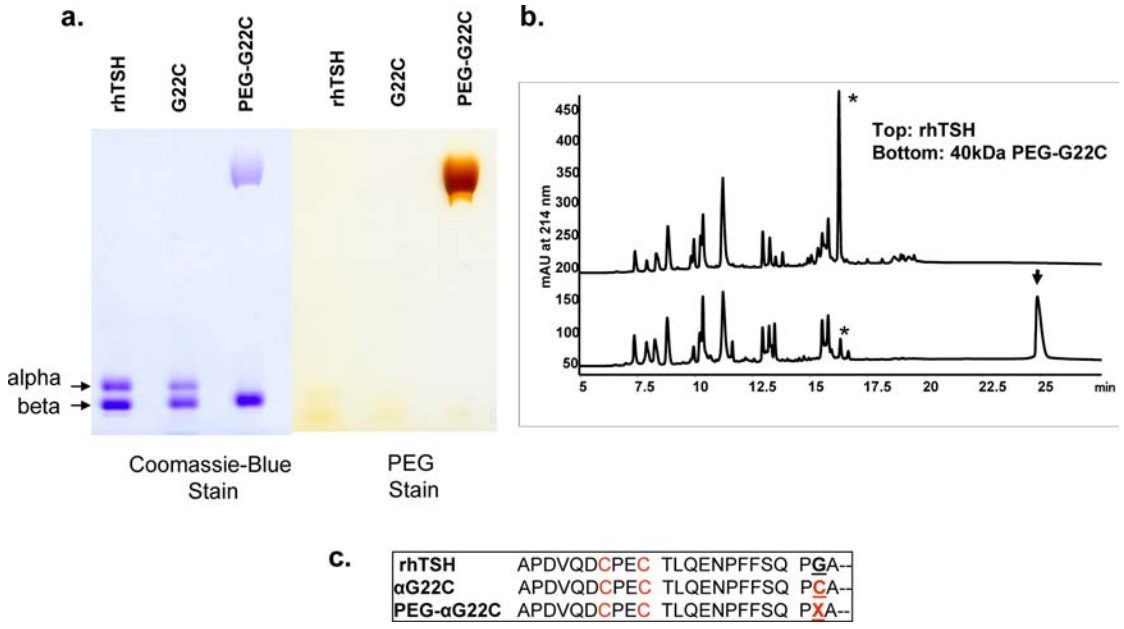

Figure 8. Confirmation of subunit- and site-specific conjugation. rhTSH, αG22C rhTSH, and 40 kDa αG22C PEG rhTSH were analyzed on a reducing SDS-PAGE gel and stained with Coomassie-blue and PEG stain (a). The positions of the subunit are indicated by arrows. Reverse-phase analysis of trypsin-digested rhTSH and 40 kDa αG22C PEG rhTSH is shown in part b. The Gly22/Cys22-containing peptides (AT1) are marked with asterisks. The PEG-attached AT1 is labeled with an arrow. The N-terminal sequencing result for the collected PEG-AT1 is shown in part c.

Table 2. Comparison of TSH PEGylation Strategies

PEGylation target	# of conjugation sites	homogeneity	protein	mono-PEGylation yield[a]	purification yield[b]	combined yield[b]
lysine	13	low	rhTSH	18%	~96%	~17%
N-termini	2	medium	rhTSH	39%	~90%	~35%
engineered cysteine	1	high	Cys Mutants	82%	~96%	~79%

[a]Representative PEGylation yield optimized for each target. For lysine PEGylation, low PEG:TSH ratio (1:1) was used to minimize formation of di-PEGylated species. For N-terminal PEGylation, a higher PEG:protein ratio (5:1) was used to increase the yield on the two termini. For cysteine PEGylation, 2 mM cysteine was used to reduce the capped G22C before the reaction where a PEG:protein ratio of 5:1 was used. [b]Single-column purification yield was used for comparisons. Additional steps such as concentration and further purification will lead to variation of the final yield.

fragments was collected and analyzed by amino acid sequencing (Figure 8c). Reliable signal was achieved for 23 cycles and indicated only the sequence of the AT1 fragment. The predominant sequence was attributed to the native N-terminus, while a minor sequence was also observed beginning at Asp3. Since no sequences other than that of AT1 were observed, these results support the selective conjugation to within the six cysteines on this fragment. For cycles 7 and 10, *S*-carboxymethylated Cys were observed as expected from the reduced and alkylated Cys residues at these two positions. Neither Cys nor *S*-carboxymethyl Cys was observed at position 22 (designated X in Figure 8c). These data suggest highly selective PEGylation at Cys 22, the targeted conjugation site. Combined with the SDS PAGE data, they indicate the successful generation of site-specific mono-PEGylated product with the conjugation site at the engineered αG22C site on the α-subunit.

DISCUSSION

In the clinic, a two-injection regimen separated by 24 h is recommended for Thyrogen administration, followed by radioiodine administration on day 3 and diagnostic scanning and thyroglobulin testing on day 5. This rigid dosing regimen is necessitated by the relatively short circulation half-life of rhTSH. This two-dose course of therapy is also aimed at reducing potential undesired side effects due to the high peak-to-trough plasma exposure. The work reported here aims to produce a long-acting TSH with sustained exposure after a single injection through conjugation to PEG polymers.

MonoPEGylated proteins are often desired over generally less active multiPEGylated species. The separation of monoPEGylated species from multiPEGylated and unPEGylated species is an important prerequisite to developing a controlled process for manufacturing scale-up. Amine-specific PEGylation of lysines and N-termini of rhTSH resulted in low reaction yields of mono-PEGylated products due to multiple targets for conjugation—13 exposed lysines or 2 available N-termini. Lower pHs required for more selective N-terminal PEGylation[10] were not compatible with rhTSH stability (data not show), thus leading to a compromise of pH 5.6 that resulted in significant side conjugations. The presence of two or more target conjugation sites with similar conjugation propensity would invariably lead to <50% reaction yield for mono- or multi-PEGylated species independent of PEG:protein ratio. With multiple potential conjugation targets, low PEG:TSH ratios would yield predominantly monoPEGylated conjugates, but at the expense of more unconjugated rhTSH (Figure 2c), while high PEG:TSH ratios would yield significant amounts of multiPEGylated species (Figure 2d) that would be difficult to separate from monoPEGylated species. Although the reaction yield of monoPEGylated species could be higher at

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high PEG:TSH ratios (Figure 2), the final yield of purified monoPEGylated species was typically under 15% (Table 2). In addition to the yield issue, amine-PEGylated rhTSH also displayed over 14.6−36.4-fold loss in receptor binding (Table 1). While this may not be surprising for lysine conjugates because many lysines are at the interface with the receptor (Figure 1), this is somewhat unexpected for the N-terminal conjugates, given that they are positioned away from the receptor binding interface. However, the crystal structure of FSH-FSHR complex only contains part of the receptor.[18] It is possible the C-terminal portion of TSHR not shown in our model (Figure 1) can wrap around TSH and contact the N-termini. These limitations observed for PEGylation of the wild-type TSH, together with the difficulties expected in process control and product characterization (Table 2), led us to pursue a strategy for site-specific mono-PEGylation by introducing single cysteines into rhTSH.

Although PEGylation through engineered Cys is well-documented in the literature,[7,16,27] creating rhTSH Cys variants poses multiple challenges. First, TSH is a small hormone with an extensive receptor contact interface.[1] Second, TSH belongs to the "cysteine-knot" family of proteins with extensive disulfide bonds in both subunits (10.5% of amino acids are cysteines, Figure 1). When choosing sites for Cys mutagenesis, amino acids close to existing disulfide bonds may cause disulfide bond scrambling and consequently protein unfolding. We designed a series of Cys mutants based on our TSH/TSHR structure model for minimal perturbation on the disulfide bonds, yet half of the mutants had either no (βN23C, αN52C) or very low expression levels (βT21C, βD56C, and βE63C) (Figure 2a), highlighting the importance of screening a large number of mutants. The loss expression for αN52C, αN78C, and βN23C may be due to the loss of carbohydrates, consistent with the lower expression reported for glutamine mutants at these positions.[28] One mutant, αM71C, expressed at a relatively high level, but appeared to exist in higher-order forms, presumably due to oligomerization through the unpaired Cys introduced or through scrambled disulfide bonds. Our work suggested that cysteine-knot proteins pose additional challenges for site-specific conjugation via engineered cysteines.

Another interesting phenomenon observed for the Cys variants is the "capping" of the engineered Cys, which we effectively removed ("decapped") by using cysteine as a mild reductant. This has been previously documented in other free Cys-containing proteins, with cysteinylation and glutathionylation demonstrated to be the major cause of modification.[25] This capping seemed to effectively block the reactivity of the otherwise free thiol group toward malPEGylation; without reduction, the PEGylation yield was only around 16% (Figure 5b). It is particularly challenging in this work due to the presence of the cysteine-knot structure: the cap on cysteines needs to be removed by reduction for PEGylation without reducing the disulfide bonds. Initial reduction experiments indicated that TCEP and DTT effectively reduced the modified Cys, but also reduced other disulfide bonds to generate nonspecific PEGylation. A longer incubation after removal of the reductants helped to lower the percentage of multi-PEGylated species (data not shown), suggesting refolding of some reduced disulfide bonds. Even with extensive titrations and refolding, >5% of the multi-PEGylated rhTSH was still present in the TCEP reduced rhTSH PEGylation reaction. Interestingly, 2 mM cysteine had the balanced effect of removing the cap from the engineered Cys, with limited effect on the existing disulfide bonds in the structure (Figure 5). Similar conditions have been previously used by Banks et al. for an unpaired cysteine residue found in the variable domain of a recombinant human IgG antibody.[24] Combined with mono-S column purification, a homogeneous mono-PEGylated αG22C was obtained. The loss of the α-subunit after PEGylation seen on the Coomassie-blue stained SDS-PAGE under reducing conditions (Figure 8) confirms that PEGylation occurred on the α-subunit. This is further confirmed by N-terminal sequencing of the PEGylated αG22C which showed the lack of reaction at the αG22C position. We have demonstrated the successful site-specific mono-PEGylation with this cysteine-knot protein.

It is well-documented that PEGylation can reduce the *in vitro* biological function of therapeutic proteins,[29] but this reduction seems to be more than compensated by gains in *in vivo* efficacy due to prolonged duration of action. For example, the *in vitro* antiviral activity of PEGylated interferon α-2a was found to be 7% of the original activity. In contrast, the *in vivo* antitumor activity was several-fold enhanced compared to interferon α-2a.[9] The work reported here appears to be consistent with these prior observations. We developed an ELISA-based competition receptor binding assay to compare the IC_{50} of rhTSH, αG22C rhTSH, and various malPEG products. The αG22C mutant itself had little effect on its ability to bind the TSH receptor (Figure 6), consistent with the structure model which suggests that Gly22 sits in a loop in the α-subunit, away from the receptor binding interface (Figure 1). Adding the PEG group did reduce the binding affinity by 4.6−15.9-fold (Table 1). The conjugation site on the engineered Cys22 is on the opposite side of the receptor binding interface in our structural model (Figures 1 and 3). Given the small size of rhTSH and the flexible PEG polymers, it is possible that the PEG can wrap around the rhTSH molecule and reach the binding interface. The other possibility is that the binding interface with the full-length TSH receptor may be larger than our structure model predicts. A bigger PEG (60 kDa) seems to lead to an even lower affinity compared to 40 kDa αG22C PEGs as expected.

TSH causes the thyroid gland to make two hormones: triiodothyronine (T3) and thyroxine (T4). We developed a rat pharmacodynamic model to evaluate the TSH *in vivo* function by monitoring the circulating level of T4, which is solely a result of rhTSH administration as implanted T3 suppresses the autogenous generation of T4 during the test period. The result from this pharmacodynamic test in rats has shown a prolonged duration of action beyond 24 h with the 40 kDa αG22C PEG rhTSH (Figure 7). This provides another example that the loss of *in vitro* activity can be more than compensated with extended duration of reaction *in vivo*. Given the partial loss of *in vitro* binding activity, this extended PD effect is likely a result of prolonged circulation time, which we have observed in this rat model for a 40 kD PEG-rhTSH conjugate produced by an alternative carbohydrate-based conjugation chemistry (Park et al., unpublished). The T4 level stimulated by 40 kDa αG22C PEG-rhTSH is actually lower at early time points compared to that of rhTSH presumably due to a slower distribution of the bulky conjugate from tissues upon intramuscular injection. This lower peak-to-trough T4 level induced by 40 kDa αG22C PEGrhTSH may lead to reduced side effects compared to rhTSH.

Hu et al. have previously reported engineering cysteines into another Cys-rich protein BMP-2.[30] Their results indicate that specific attachment of PEG at engineered sites of BMP-2 is

possible and that the attachment site is critical for biological activity. The work reported here demonstrates the successful site-specific Cys PEGylation of another multisubunit cysteine-knot protein. In addition to a more controlled process that generates a more homogeneous product profile compared to amine-based PEGylation, Cys PEGylation leads to higher yields of mono-PEGylated rhTSH (Table 2), since we can drive the reaction to completion without generating significant amounts of multi-PEGylated products. This overcomes the challenges encountered by lysine and N-terminal PEGylation due to the presence of multiple conjugation targets, as demonstrated in the reaction yield differences (85% vs 20−50%). Furthermore, the purification yields for Cys conjugates are much higher (90% vs 50%) because of minimal multi-PEGylated species that are difficult to separate from mono-PEGylated species, especially at larger scales.

This work is another demonstration of successful site-specific PEGylation of a cysteine-knot protein by an engineered cysteine mutation. The PEGylated product was shown to remain biologically functional *in vivo* with prolonged duration of action and lower peak-to-trough T4 response and may provide clinical benefit for more convenient dosing and a better therapeutic index.

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Notes

The authors declare no competing financial interest.

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