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Cysteine-Specific PEGylation of rhG-CSF via Selenylsulfide Bond

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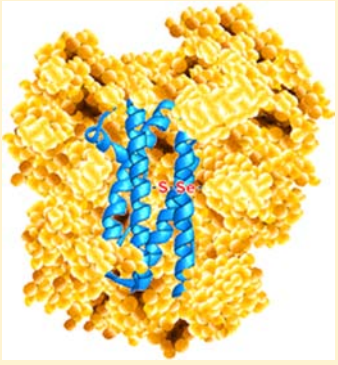
Supporting Information

ABSTRACT: A new PEGylation reagent enabling selective modification of free thiol groups is described in this article. The reagent was synthesized by attaching linear polyethylene glycol (PEG) *N*-hydroxysuccinimide to selenocystamine. The reaction was very fast, resulting in over 95% conversion yield. The active group of this new PEG-Se reagent is a diselenide, reacting with thiols via thiol/diselenide exchange reaction. Recombinant human granulocyte colony-stimulating factor (rhG-CSF) with an unpaired cysteine at the position 18 (Cys18) was used as a model protein. It was comparatively PEGylated with the new PEG-Se reagent, as well as with commercially available maleimide (PEG-Mal) and *ortho*-pyridyl disulfide (PEG-OPSS) PEG reagents. The highest PEGylation yield was obtained with PEG-Mal, followed by PEG-OPSS and PEG-Se. The reaction rates of PEG-Mal and PEG-Se were comparable, while the reaction rate of PEG-OPSS was lower. Purified monoPEGylated rhG-CSF conjugates were characterized and compared. Differences in activity, stability, and *in vivo* performance were observed, although all conjugates contained a 20 kDa PEG attached to the Cys18. Minor conformational changes were observed in the conjugate prepared with PEG-Mal. These changes were also reflected in low *in vitro* biological activity and aggregate formation of the maleimide conjugate. The conjugate prepared with PEG-Se had the highest *in vitro* biological activity, while the conjugate prepared with PEG-OPSS had the best *in vivo* performance.



INTRODUCTION

PEGylation has become an established method for improving therapeutic properties of protein drugs.[1,2] There have been several very successful PEGylated drugs on the market for more than two decades, exploiting PEGylation for the improvement of *in vivo* elimination half-life. Poor solubility, immunogenicity, and stability issues of proteins can also be successfully solved by PEGylation.[3] Neulasta, a site-specifically monoPEGylated recombinant human granulocyte colony-stimulating factor (PEG-rhG-CSF/pegfilgrastim), used for the treatment of chemotherapy induced neutropenia, is a good example of the benefits achieved by PEGylation. PEGylation has improved the *in vivo* elimination half-life of pegfilgrastim to such an extent that only one dose per chemotherapy cycle can be given instead of up to 14 daily injections.[4−6] The shelf life stability of pegfilgrastim was also improved by slowing down the aggregation kinetics.[7]

A current trend in PEGylation technology is to optimize PEG−protein conjugates by protein engineering, in order to promote site-directed monoPEGylation, and/or by designing new chemical linkages between the polyethylene glycol (PEG) and the protein. Using classical amino-PEGylation chemistries, well-defined conjugates with site specific PEG-attachment are not easily prepared, due to the usually high number of surface exposed lysines. An improved PEGylation selectivity can be achieved by reductive alkylation, which more selectively attaches PEG to the N-terminal amino acid.[6,8] Some new technologies for site-directed PEGylation, which employ chemical or enzyme-mediated conjugation to specific or unnatural amino acids, have also been described.[9]

Cysteine-specific PEGylations with maleimide (Mal), vinyl sulfone, *ortho*-pyridyl disulfide (OPSS), thiol, or iodoacetamide activated PEGs belong to the first approaches used for site-directed PEGylation. The unpaired cysteine residue is not very frequent in the protein structure, especially not on the surface. In the absence of free cysteines, they can be genetically engineered into the amino acid sequence at appropriate sites,[10] or free thiol groups can be introduced through chemical modification of the amino groups.[11] Cysteine PEGylation using OPSS (protected thiol) or thiol-activated PEG reagents offers an additional advantage of reversible PEGylation by forming a disulfide bond between the protein and PEG. The disulfide bond is a stable covalent bond in the normal environment and can be cleaved in the reductive environment. A large difference in the redox potential between the oxidizing extracellular space and the reducing intracellular space makes the disulfide bond a very interesting tool for designing conjugates that can release native protein at a desired location in the body.[12] Reversible PEGylation may offer the highest benefit to small molecules where the *in vitro* biological activity can almost be diminished

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because of the bulky PEG. Such examples are peptides, oligonucleotides, analogues of protein domains, and small molecular weight drugs (e.g., anticancer drugs). Lower PEG accumulation is also anticipated in the case of reversible PEGylation techniques. The cleaved PEG chains are more easily eliminated from the body than the whole PEG−protein conjugates. PEG accumulated in the cells may induce some undesirable side effects, especially in the long term use of PEGylated proteins, although PEG toxicity issues have not been frequently addressed in the literature.

A selenylsulfide bond can be formed between selenol and thiol,[13] by analogy to a disulfide bond formation between two thiols. Selenium and sulfur are the elements of the same group in the periodic table. They display many similarities, as well as some differences.[14] A higher reactivity of selenol compared to thiol and a lower reduction potential of diselenides and selenylsulfides compared to the disulfides suggest that a superior coupling reagent could be obtained by introducing selenium into the reactive group. Replacement of sulfur with selenium has already been exploited in various applications.[15−20]

Human granulocyte colony-stimulating factor (hG-CSF) is a glycoprotein with a typical cytokine structure. A four helix bundle is stabilized by two disulfide bridges. It contains an additional, nonpaired, partially solvent exposed cysteine at position 17 (Cys17).[21−23] As a hematopoietic regulator of the neutrophil lineage, hG-CSF controls the differentiation of precursor cells to the neutrophils, their proliferation, and stimulates the release of mature neutrophils from the bone marrow.[24,25] The recombinant form of the protein (rhG-CSF) expressed in *Escherichia coli* (*E. coli*) is nonglycosylated and has been successfully used for the treatment of chemotherapy-induced neutropenia. A PEGylated form of rhG-CSF was introduced to the market in 2002 and has a better pharmacokinetic profile than rhG-CSF. The free cysteine in the rhG-CSF molecule has already been used as a conjugation site for the attachment of different thiol reactive reagents.[26−28] The conjugates obtained with PEG-OPSS, PEG-Mal, and PEG-vinyl sulfone under partially denaturating conditions displayed rapid aggregation. A specially designed reagent with a hydrophobic insertion was also used for the PEGylation of the free cysteine to reduce the rhG-CSF tendency for aggregation.

This paper presents the preparation of a very simple PEGylation reagent with a diselenide in the reactive group, capable of forming the selenylsulfide bond with a free cysteine of the protein. rhG-CSF with a free cysteine at position 18 (Cys18) was used as a model molecule. Besides the rhG-CSF conjugate, prepared with a new PEG-Se reagent, conjugates with PEG-Mal and PEG-OPSS were also prepared for comparison. The reactivity of all three PEGylation reagents was assessed. Furthermore, pure monoPEGylated conjugates were isolated and characterized by various analytical techniques. The effect of polymer attachment to the rhG-CSF via various linkers was studied by determination of physicochemical and biological properties of the conjugates. The rhG-CSF conjugates prepared by PEG-OPSS and PEG-Se were also evaluated *in vitro* and *in vivo* for their ability to release the native rhG-CSF.

■ MATERIALS AND METHODS

rhG-CSF (filgrastim) was produced in *E. coli* as described earlier.[29−31] Neulasta[32] (rhG-CSF with 20 kDa PEG attached to the N-terminus) was purchased from Amgen. A 20 kDa PEG with *N*-hydroxysuccinimide reactive group (PEG-NHS) and a 20 kDa PEG with maleimide reactive group (PEG-Mal) were purchased from NOF (Japan). A 20 kDa PEG with *ortho*-pyridyl disulfide active group (PEG-OPSS) was purchased from Creative PEGworks (USA). Human G-CSF ELISA was purchased from IBL International (Germany). SAMSA fluorescein (A-685) was purchased from Invitrogen.

HPLC Analyses. HPLC analyses were performed on the Agilent 1200 system. The formation of PEG with diselenide reactive group (PEG-Se), PEGylation of rhG-CSF, isolation of PEG-conjugates, and PEG-cleavage were monitored with reversed phase chromatography (RP-HPLC). RP-HPLC was carried out on YMC-ODS-AQ, 4.6 mm × 15 cm column (YMC, Japan) with mobile phase A 10% acetonitrile (ACN), 0.1% trifluoroacetic acid, mobile phase B (MPB) 90% ACN, 0.1% TFA, and a linear gradient from 46% to 70% MPB in 30 min, at 1 mL/min, 65 °C. For monitoring the PEGylation reaction of the deprotected SAMSA fluorescein, the same mobile phases were used with a linear gradient from 10% to 100% MPB in 17 min, at 1 mL/min.

Synthesis of Selenium Containing PEGylation Reagent (PEG-Se). PEG-Se reagent was synthesized in a two-step procedure. 350 mg of 20 kDa PEG-NHS reagent was added to 3.19 mg selenocystamine dissolved in 200 mM Na-phosphate pH 8.0. The mixture was stirred at room temperature, and after half an hour, additional 150 mg of PEG-NHS reagent was added. The reaction was monitored by the RP-HPLC analysis.

For the PEGylation of rhG-CSF the buffer was exchanged into the 25 mM Na-phosphate pH 7.5 using Amicon ULTRA centrifugal units with 3000 Da MWCO.

PEGylation Reaction. The PEGylation reactions were performed in a phosphate buffer pH 7.5, in the presence of a 0.05% SDS at 4 °C and without stirring. The pH of the rhG-CSF solution was adjusted to 7.5 with 0.5 M disodium hydrogen phosphate, 5% glycerol. The PEG reagent dissolved in 20 mM sodium phosphate pH 7.5 and the SDS solution were added to the protein in the amounts required for a 10-fold molar excess of PEG reagent, and 0.05% of SDS, respectively. The same PEGylation conditions were applied for the analytical scale PEGylation reactions and for the larger scale PEGylation reactions for the isolation of PEG-conjugates.

Isolation/Purification of PEGylated rhG-CSF Conjugates. SDS was removed from the PEGylation mixtures using a Dowex ion-exchange resin. Dowex was added directly into the reaction mixtures. After one hour of gentle stirring at room temperature, Dowex was removed by centrifugation. To achieve binding onto the cation exchange (CEX) column, the pH of the reaction mixture was lowered to 4.0 by a 3-fold dilution with 100 mM acetic acid.

Conjugates were isolated on a chromatographic system AKTA purifier using CEX. An 8 mL column (Tricorn 10/100) packed with SP-5PW TSK-Gel resin (TOSOHAAS) was used for the separation. The binding buffer was 25 mM sodium acetate pH 3.8. PEGylated and non-PEGylated forms of rhG-CSF were separated using a shallow linear gradient from 0% to 100% of elution buffer (75 mM sodium acetate pH 8.0) in 35 column volumes. The separation was performed at a 2.0 mL/min flow rate. The CEX fractions were pooled according to the SDS-PAGE analyses performed under nonreducing conditions (Simply Blue staining).

PEGylation of SAMSA Fluorescein. SAMSA fluorescein is a fluorescent probe with a protected thiol group. SAMSA fluorescein was treated with 0.1 M sodium hydroxide according to the manufacturer's protocol, to achieve deprotection of a thiol group. After deprotection, sodium hydroxide was neutralized using 6 M hydrochloric acid and buffered with 0.5 M sodium phosphate pH 7.5. Solid PEG-Se reagent was added to deprotected SAMSA fluorescein in 10- or 20-fold molar excess. The analytical scale PEGylation reactions were monitored at 4 °C for 10 h, using RP-HPLC analysis with fluorescent detector. The first analysis was performed after 5 min and the following analyses every hour.

***In Vitro* Biological Activity.** *In vitro* biological activity measurements of rhG-CSF are based on the stimulation of cell proliferation of the mouse myeloid leukemic cell line M-NFS-60. Briefly, the cells were seeded on microtiter plates. Then serial dilutions of WHO reference standard (NIBSC, 88/502) and the test samples were added into the culture medium. The cells were incubated for 48 h. After incubation, the MTT reagent (3-[4,5-methylthiazole-2yl]-2,5-diphenyl tetrazolium bromide) was added. During incubation the mitochondrial dehydrogenase of live cells reduces the MTT reagent into the insoluble formazan crystals. The crystals were then solubilized and the absorbance at 570 nm measured. The absorbance values represented a natural logarithm of the cell concentration. The test sample's activity was determined by comparing the dilutions of the reference standard and the test sample at which 50% of maximal proliferation stimulation was achieved.

Circular Dichroism (CD) Studies. The CD spectra were recorded in the far UV spectrum (200−260 nm). The concentration of conjugates for the analysis was 0.2 mg/mL in 10 mM Na-acetate pH 4.0 buffer. The analysis was performed on the Chirascan CD Spectrometer using 1 mm path length quartz cells. The spectral data are expressed as molar ellipticity calculated using a mean residue weight of 107.4.

Pharmacokinetic and Pharmacodynamic (PK/PD) Studies in Rats. All rats received care in compliance with the European Convention for the Protection of Vertebrate Animals and European Directive 86/609/EEC. The study was approved by the Veterinary Administration of the Republic of Slovenia. Four animals were included in each study. 50 μg (250 μg/kg) of each sample was applied subcutaneously and blood samples were taken at appropriate time intervals. The contents of rhG-CSF and its PEGylated conjugates in serum at different time points were measured using the ELISA test. The serum concentration of each conjugate was calculated from the standard curve, which was generated using the same conjugate as it was investigated in the rats. The assay was performed following the manufacturer's instructions.

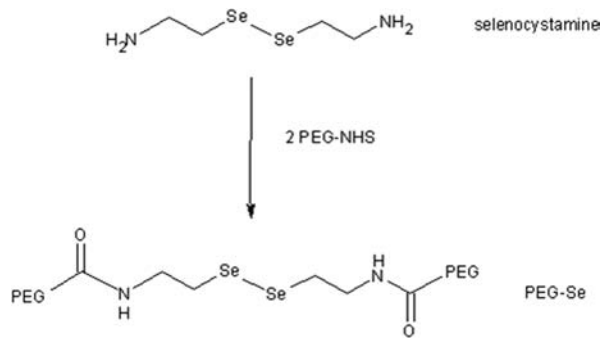
The number of leukocytes was determined in the whole blood using a hematological analyzer ABC Vet (ABX Diagnostics, France) and the percentage of the neutrophil count was determined manually under the light microscope. The absolute neutrophil count (ANC) was calculated by multiplying the total leukocyte count and the percentage of neutrophils.[33] The obtained results represent PK and PD profiles of the tested samples.

***In Vitro* PEG Release Study.** The *in vitro* PEG release studies were performed by incubating the PEG-conjugates at 0.01 and 10 mM concentration of reduced glutathione (rGSH), thus mimicking the redox potential of the extracellular and the intracellular matrix, respectively. The experiments were performed in sodium phosphate buffer pH 7.4. The amount of the released rhG-CSF was determined by RP-HPLC analysis.

RESULTS

Synthesis of the PEG-Se Reagent. The synthesis of the PEG-Se was performed by coupling the commercial PEG-NHS reagent and selenocystamine. The reaction is presented in Scheme 1. The resulting reagent is composed of two PEG moieties as a result of selenocystamine being a symmetrical molecule, with two amino groups available for conjugation. The reaction was performed in an aqueous solution where only 1.25-fold molar excess of PEG-NHS per amino group was needed for more than 95% conjugation. Buffer exchange was performed in order to remove the residual nonreacted selenocystamine. The nonreacted PEG-NHS reagent hydrolyzes in aqueous solutions to nonreactive PEG-carboxylic acid. Therefore, it was not removed.

Scheme 1. Synthesis of PEG-Se



Analytical Scale PEGylation Reactions. PEGylation reactions of rhG-CSF with the PEG-Se and with the commercial PEG-OPPS and PEG-Mal reagents were performed on an analytical scale in order to evaluate the differences in the conjugation kinetics. The analytical scale PEGylation reactions were monitored at 4 °C for 20 h, using RP-HPLC analysis. The first analysis was performed after 30 min and the following analyses every two hours. PEG-Se reacted with the rhG-CSF very quickly. After 30 min 29% of the conjugate was formed. Afterward, the reaction almost stopped and the final PEGylation yield reached 38%. The PEG-OPSS reagent reacted with rhG-CSF more slowly. The final PEGylation yield after 20 h was 64%. The PEG-Mal reagent resulted in the highest reaction yield, reaching 93% (Figure 1A).

The comparison of the PEG-Se conjugation kinetics at 10, 20, and 30-fold molar excess of the reagent, revealed the same reaction pattern. The initial part of the reaction was very fast and afterward the conjugation reaction almost stopped. Although the reaction pattern was the same, with almost the same time points of the decelerated reaction, higher PEG excesses resulted in higher yields. The 30-fold PEG excess resulted in an 84% conversion yield, while the 10-fold PEG excess was sufficient for only 32% conversion yield. The conjugation kinetics is shown in Figure 1B.

In the reaction of PEG-Se with deprotected SAMSA fluorescein two different kinetics are present (Figure 1C). In the first minutes the reaction is very fast and afterward significantly slower. By using a 10× molar excess of PEG-Se reagent, 60% of the conjugate is formed almost instantly. On the other hand to achieve 100% conversion with 10-fold molar excess of PEG-Se reagent almost 10 h are needed. 20-fold

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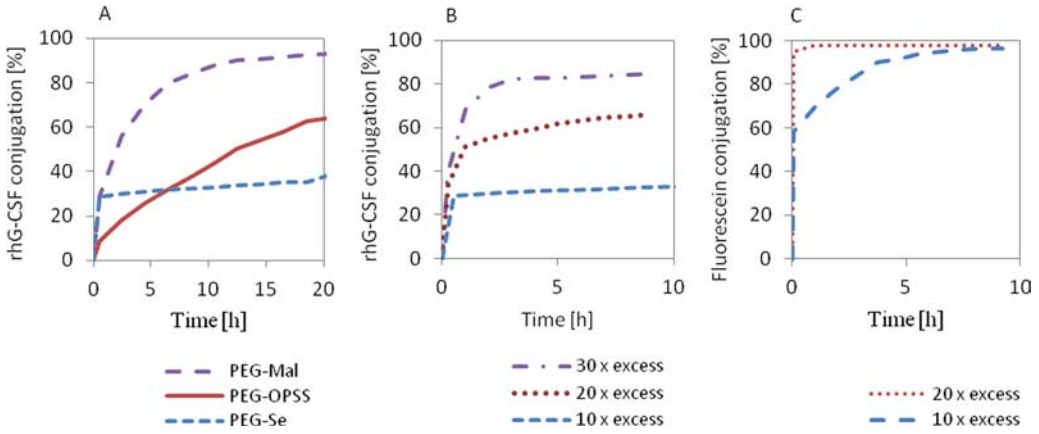

Figure 1. Time courses of the rhG-CSF PEGylation profiles using 10-fold molar excess of PEG-Mal, PEG-OPSS, and PEG-Se (A), time courses of the rhG-CSF PEGylation profiles using 10, 20, and 30-fold molar excess of PEG-Se (B), and time courses of the SAMSA fluorescein PEGylation using 10 and 20-fold molar excess of PEG-Se (C).

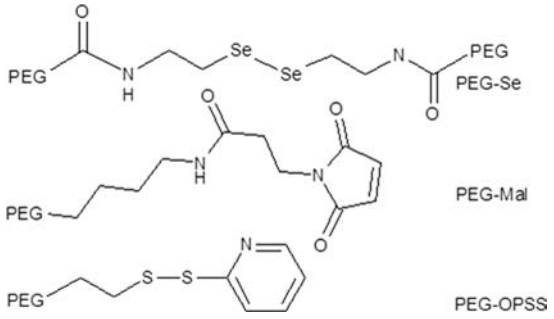

Figure 2. Structure of PEG-reagents.

molar excess of PEG-Se provides complete conjugation of SAMSA fluorescein in 30 min.

Characterization of Conjugates. For characterization purposes the rhG-CSF conjugates were prepared on a preparative scale. The purified conjugates prepared using PEG-Mal, PEG-OPSS, and PEG-Se reagents, were designated as FC18-Mal-20L, FC18-OPSS-20L, and FC18-SeC-20L, respectively. Structural properties of the prepared conjugates are summarized in Table 1.

Table 1. Structural Properties of the Molecules Used in the Experiments

conjugate	PEGylation reagent	conjugation site	linker stability in reducing environment
Neulasta	PEG-CHO	Met1	stable
FC18-Mal-20L	PEG-Mal	Cys18	stable
FC18-OPSS-20L	PEG-OPSS	Cys18	cleavable
FC18-SeC-20L	PEG-Se	Cys18	cleavable

SDS-PAGE analyses were performed using nonreducing conditions with protein-specific staining. Small differences in the molecular weight observed on the SDS-PAGE gels are a consequence of slight MW differences among the starting PEG reagents (Figure 3A).

RP-HPLC and **SE-HPLC** purities of the PEG-conjugates were not the same, which can be seen in Table 2 and Figure 3. The RP-HPLC purity of the FC18-Mal-20L conjugate was the highest, while the same conjugate exhibits the lowest SE-HPLC purity. The FC18-SeC-20L exhibits the lowest RP-HPLC purity and a rather low SE-HPLC purity.

Far UV-CD spectra of rhG-CSF and the prepared conjugates are shown in Figure 4. The superimposition of the spectra indicates that the conjugation procedure did not cause any major change in the secondary structures. Minor differences in the second minimum can be observed for the FC18-Mal-20L conjugate (Figure 4, inset).

***In vitro* biological activities (BA)** of the conjugates are presented in Table 2. All the prepared conjugates expectedly exhibited lower *in vitro* BA compared to the native rhG-CSF. Surprisingly high differences between the conjugates bearing different 20 kDa PEGs attached to the same Cys18 attachment site were observed. BA ranged from 55% for the FC18-SeC-20L conjugate, followed by 19% for the FC18-OPSS and 5% for the FC18-Mal-20L.

***In vitro* PEG release study** was performed to evaluate differences in the release of the native protein from the rhG-CSF conjugates. As seen in Figure 5, the rhG-CSF was already generated from the FC18-SeC-20L conjugate at 0.01 mM rGSH concentration (at pH 7.4). In contrast, the FC18-OPSS-20L containing a disulfide bond was not cleaved, either at 0.01 mM rGSH concentration or at 10 mM rGSH concentration at pH 7.4. The reduction of FC18-SeC-20L conjugate at 10 mM rGSH concentration was almost instant. An efficient release of rhG-CSF from the FC18-SeC-20L conjugate was also obtained at 10 mM rGSH concentration, pH 4.0, although the release was slower.

***In vivo* study** was performed in rats in order to generate PK and PD profiles (Figure 6). Native rhG-CSF and conjugates were administered in single dose subcutaneously. The PK profile of the FC18-SeC-20L was somewhat similar to the PK profile of the native rhG-CSF, while the PK profile of the FC18-OPSS-20L was similar to the PK profile of Neulasta. The PK profile of the FC18-Mal-20L conjugate was unexpected. The elimination half-life was almost as high as for Neulasta and the FC18-OPSS-20L, but C_{max} was significantly lower. The elimination half-lives of the conjugates are presented in Table 2.

The PD profiles are shown in Figure 6. The PD profiles of rhG-CSF and FC18-SeC-20L were similar. The highest AUC was obtained for Neulasta. The FC18-OPSS-20L displayed a lower AUC and the FC18-Mal-20L a significantly lower AUC.

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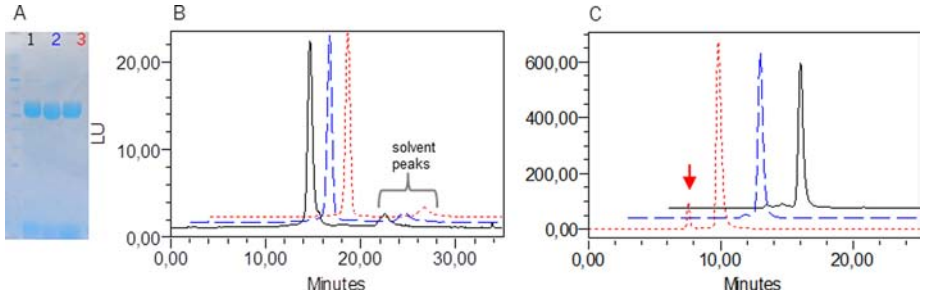

Figure 3. SDS-PAGE analysis (A), RPC analysis (B), and SEC analysis (C) of rhG-CSF conjugates (1 or solid line: FC18-SeC-20L, 2 or dashed line: FC18-OPPS-20L, 3 or dotted line: FC18-Mal-20L). For a better visualization an offset in *X* and *Y*-axes was applied. Soluble aggregates seen in FC18-Mal-20L conjugate are marked with an arrow.

Table 2. Characterization Results of rhG-CSF Conjugates

protein/ conjugate	residual *in vitro* BA (%)	RPC purity (area %)	SEC purity (area %)	elimination half-life (h)
rhG-CSF	100	97.8	97.2	2.1
Neulasta	45	96.0	98.1	7.7
FC18-OPSS-20L	19	93.6	96.3	7.2
FC18-SeC-20L	55	92.9	93.3	1.6
FC18-Mal-20L	6	97.5	91.9	6.5

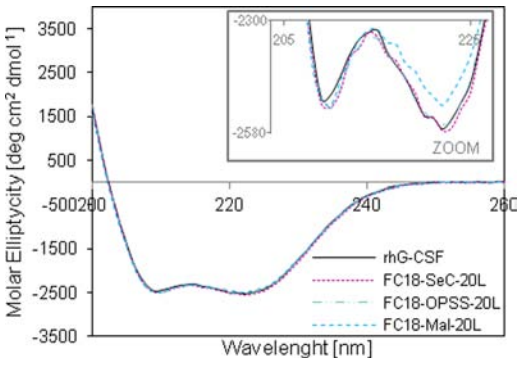

Figure 4. Far UV-CD spectra of rhG-CSF and rhG-CSF conjugates.

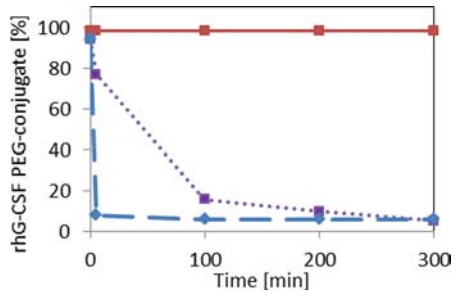

Figure 5. Time course of PEG release from the rhG-CSF conjugates (solid line, FC18-OPSS-20L incubated with 0.01 mM or 10 mM rGSH at pH 7.4; dotted line, FC18-SeC-20L incubated with 0.01 mM rGSH at pH 7.4; dashed line, FC18-SeC-20L incubated with 10 mM rGSH at pH 7.4).

DISCUSSION

A PEGylation reagent should be selective and highly reactive, and should enable high PEGylation yields. Cysteine specific PEGylation seems to be ideal for achieving these criteria.

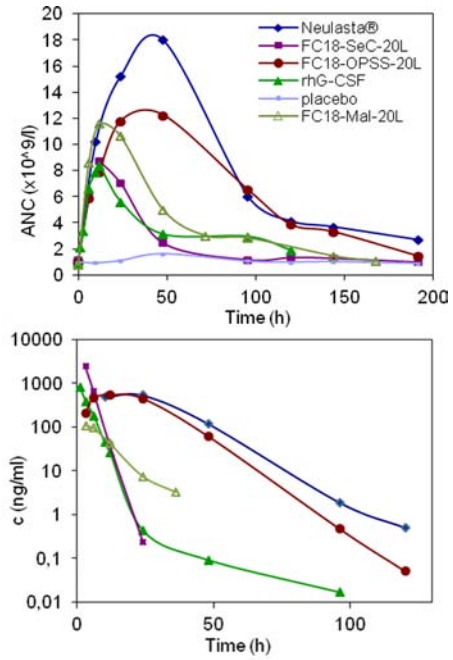

Figure 6. PD profiles (upper panel) and PK profiles (lower panel). ANC: absolute neutrophil count.

Although some PEGylation reagents on the market fulfill the above-mentioned requirements, all the possibilities for thiol PEGylation have not been tested yet. It has been shown in other applications that the replacement of sulfur with selenium produces a superior molecule with higher reactivity. We have prepared a new cysteine-specific PEGylation reagent by simply attaching PEG-NHS to the diselenide containing selenocystamine molecule. This kind of reaction is usually performed in organic solvents due to the NHS group instability in water. In our case the reaction between the NHS group and the amino group of the selenocystamine happened in a few minutes, so that the NHS group instability was not an issue and a high conversion yield could be achieved with low excess of PEG-NHS. Transfer of the reaction into an organic solvent, where the reaction rate is normally much slower, was not necessary.

The new PEG-Se reagent was tested on rhG-CSF which is an important therapeutic protein, either as a sole protein or in the PEGylated form Neulasta. Neulasta, the N-terminally PEGylated rhG-CSF, has been on the market since 2002. In the last ten years several other improved, long-acting variants of the modified rhG-CSF have also been reported, reaching various phases of technical and clinical development.[34−39] With

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its partially exposed free cysteine, the rhG-CSF offers an ideal possibility for cysteine specific PEGylation. Therefore, we have prepared the Cys18 PEGylated rhG-CSF conjugates with our new PEG-Se reagent and with two commercial PEGylation reagents. A 20 kDa molecular weight was chosen for all the PEGylation reagents, as it is known that higher PEG molecular masses do not further increase the elimination half-life of rhG-CSF. Additionally, the commercially available PEGylated rhG-CSF also bears a 20 kDa PEG, although attached to the N-terminus.

In the case of all three reagents the start of the reaction is initiated by the nucleophilic attack of the protein thiolate on the PEG-reactive group: in the case of PEG-Se on the diselenide bond, in the case of PEG-OPSS on the disulfide bond, and in the case of PEG-Mal on the carbon adjacent to the maleimide double bond. The conjugation of PEG-Se with rhG-CSF was very fast and comparable to the PEG-Mal reagent, while the conjugation of PEG-OPSS with rhG-CSF was slower. The higher reactivity of PEG-Se over PEG-OPSS was expected due to higher nucleophilicity of selenol compared to the thiol. Both reagents, PEG-Se and PEG-OPSS, undergo a similar thiol exchange reaction. The PEG-OPSS reagent undergoes a thiol/disulfide exchange reaction with the formation of a single mixed disulfide and a nonreactive pyridine-2-thiol. The PEG-Se reagent undergoes a thiol/diselenide exchange reaction with the formation of a selenylsulfide and a reactive selenol, which probably very quickly oxidizes with another selenol molecule into PEG-Se. Probably, the reactivity of the PEG-Se reagent could be further improved by redesigning the PEG-Se reagent in order to achieve more OPSS like structure. By omitting one PEG chain and creating an asymmetrical diselenide, steric hindrance caused by two PEG moieties would be greatly reduced. Such a change would also disable the reoxidation reaction of two selenols into PEG-Se, which interferes with the conjugation reaction with protein.

In spite of the very fast reaction rate in the beginning, the PEGylation yield achieved with the PEG-Se reagent was low. 30% of the conjugate was formed very rapidly, but afterward the reaction almost stopped. By increasing the molar excess of PEG-Se reagent, the conversion yield increased, but the PEGylation pattern stayed the same: fast conversion at the beginning, followed by an almost blocked reaction. Actually, it is somehow unexpected that the sterically hindered PEG-Se reagent bearing two 20 kDa PEG chains would react more rapidly than the single PEG chain reagents PEG-Mal and PEG-OPSS. To evaluate the reactivity rate of the PEG-Se itself, avoiding the steric hindrance phenomena intrinsic of proteins, the PEG-Se was coupled with a deprotected SAMSA fluorescein and the reaction monitored with RP-HPLC using a fluorescence detector. Although SAMSA fluorescein is a small molecule, the reaction kinetics remained the same: a very fast initial part, followed by a significantly slower phase. Taking into account that the novel PEG-Se reagent contains a low percentage (less than 5%) impurity in the form of monopegylated selenocystamin, and the reagent is used in high excess, this impurity might be responsible for high initial reactivity rate. We might speculate that the sterically hindered PEG-Se with two PEG chains is responsible only for very slow later conversion, which is another reason to redesign the diselenide PEG reagent.

An intact protein structure is essential for its function. Disruption of the protein structure by PEGylation is not acceptable since it can lead to the loss of biological activity and reduced shelf life stability. rhG-CSF has an unpaired cysteine at position 18, which is buried in the hydrophobic pocket just below the protein surface and only partially accessible to the solvent. Some studies showed that the unfolding/refolding procedure in guanidinium chloride was needed for its successful conjugation.[26] In another case, a special reagent was synthesized, with a hydrophobic part next to the reactive end group, to access Cys18 under native conditions.[27] In our case the PEGylation reaction was performed in the presence of 0.05% SDS. It seems that 0.05% SDS loosens the rhG-CSF structure, opens the hydrophobic pocket, and thus makes Cys18 accessible to the PEGylation reagent. The selected PEGylation conditions with SDS do not reduce the *in vitro* biological activity of the protein. In general, the PEGylated proteins show lower *in vitro* biological activity compared to native proteins.[40] The reason for this reduction is the steric hindrance caused by the bulky PEG chain during receptor binding. *In vivo*, this effect is compensated by a substantially increased residence time in the body, which generally makes PEGylated proteins superior to native proteins. Veronese and colleagues showed that, in the case of rhG-CSF Cys18 PEGylation, the *in vitro* biological activity is reduced not only because of the steric hindrance effect, but presumably also due to some structural changes caused by the PEG attachment to the thiol buried in the hydrophobic pocket.[26] In our experiments the *in vitro* biological activities of the prepared conjugates differed significantly depending on the chosen PEGylation reagent. The lowest *in vitro* biological activity was determined for the conjugate prepared with a large maleimide reactive group, which evidently causes some changes in the rhG-CSF local structure. According to the *in vitro* biological activity results, the PEG-Se caused the lowest change in the protein structure. As confirmed by the Far UV-CD spectra, the overall secondary structure of the rhG-CSF conjugates was very close to that of the native rhG-CSF. In the CD spectra some minor changes were observed only for the FC18-Mal-20L conjugate, which is also in agreement with its low *in vitro* bioactivity and tendency to aggregate. Based on these results, it can be speculated that PEG-Mal is not the best choice for naturally present cysteines that are usually partially buried in the protein structure. In contrast, the maleimide chemistry has been proven many times as an appropriate choice for genetically introduced surface exposed cysteines.

PEGylation via formation of a disulfide bond is also known as an interesting tool for designing conjugates that could release the unmodified protein in various body compartments according to their reduction potential. In theory, the conjugates containing disulfide bond should remain intact in the extracellular environment, and the disulfide bond should be cleaved upon entering the cells.[12] However, our PK and PD studies indicate that the FC18-OPSS-20L conjugate did not generate the native rhG-CSF in the bloodstream. The opposite result was found for FC18SeC-20L, where the PK profile similar to the rhG-CSF indicates the release of the native protein in the bloodstream. The PK profile of the FC18-Mal-20L conjugate was also unexpected. According to the permanent conjugation achieved with the maleimide group, the PK profile should be similar to the Neulasta PK profile. The elimination half-life is almost as long as for the Neulasta and the FC18-OPSS-20L, but the C_{max} is significantly lower (Figure 6). It can be speculated that some of the FC18-Mal-20L conjugate was lost before entering the bloodstream, most probably due to its tendency to aggregation. Almost immediately after the

preparation, soluble higher molecular weight aggregates were formed and also observed on SEC (Figure 3C), while on RPC they were either disrupted by the mobile phase or they were too hydrophobic to elute from the column. The PD profile of the FC18-OPSS-20L conjugate shows a lower AUC compared to Neulasta, due to its lower *in vitro* biological activity. The AUC of the FC18-Mal-20L is somewhere in between because of the prolonged elimination half-life, combined with low *in vitro* biological activity.

The *in vitro* PEG release studies of the FC18-SeC-20L and FC18-OPSS-20L conjugates under reductive conditions, mimicking the redox potential of the extracellular (0.01 mM rGSH) and intracellular matrix (10 mM rGSH), were performed to check the ability of the controlled release of the active protein. However, the results with both conjugates were quite unexpected. The FC18-OPSS-20L was not reduced at 10 mM rGSH concentration as expected for disulfides, but the FC18-SeC-20L was reduced even at 0.01 mM rGSH, although selenylsulfides have a lower reduction potential than disulfides and should be more stable in the reducing environment. This unusual behavior is also reflected in the PK and PD profiles of the FC18-SeC-20L, which are very similar to rhG-CSF.

Nevertheless, the cleavage kinetics can be influenced by the groups adjacent to the disulfide. Groups, such as methyl, dimethyl, or phenyl, can reduce the cleavage kinetics by increasing the steric hindrance of the disulfide group.[41,42] It is also known that polar groups in the vicinity of the selenylsulfide bond can influence the reduction kinetics of the selenylsulfides.[43,44] In the PEG-Se and PEG-OPSS reagents the linker structure between PEG chain and the cleavage site (selenylsulfide and disulfide) is not the same (Figure 2). In fact, the linker groups that can influence the reductive stability are different. To summarize, the differences in the cleavability of the prepared conjugates cannot be explained solely based on the selenylsulfide and disulfide group properties, but depend on numerous factors. For the same reason different cleavage kinetics can also be expected when the PEG-Se or PEG-OPSS reagents are used with other proteins.

CONCLUSIONS

A novel coupling chemistry for site-specific, thiol-directed modification of proteins has been described in this article. The prepared seleno-PEG reagent could be conjugated to any free cysteine. Its behavior has been tested by its coupling to rhG-CSF at cysteine 18. The conjugation was selective and fast, although higher molar excess was needed to achieve a high conversion yield. The conjugation was reversible as native rhG-CSF was generated in the bloodstream. For comparison, conjugates with commercial reagents were also prepared and characterized. The selection of the coupling chemistry strongly influenced the *in vitro* bioactivity, stability, and *in vivo* performance of the conjugates.

Selenylsulfide coupling was found to be an interesting technology which deserves further investigation and could be applicable not only for PEGylation but also for labeling and conjugation reactions between proteins, peptides, and small molecular weight drugs.

ASSOCIATED CONTENT

Supporting Information

$^{1}H-^{13}C$ HMBC spectra of PEG-Se. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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