The ELISA Detectability and Potency of Pegfilgrastim Decrease in Physiological Conditions: Key Roles for Aggregation and Individual Variability

Tao Xie1, Hui Fang1, Weiming Ouyang1, Phillip Angart1, Meng-Jung Chiang1, Ashwinkumar A. Bhirde1, Faruk Sheikh1, Patrick Lynch1, Ankit B. Shah2, Sharadrao M. Patil3, Kang Chen5, Meiuy Shen4, Cyrus Agarabi1, Raymond P. Donnelly1, Kurt Bronson1, Sarah J. Schriever2, Kristina E. Howard2, Sarah M. Rogstad3 & David M. Frucht1*

PEGylated recombinant human granulocyte colony-stimulating factor (pegfilgrastim) is used clinically to accelerate immune reconstitution following chemotherapy and is being pursued for biosimilar development. One challenge to overcome in pegfilgrastim biosimilar development is establishing pharmacokinetic (PK) similarity, which is partly due to the degree of PK variability. We herein report that commercially available G-CSF and PEG ELISA detection kits have different capacities to detect pegfilgrastim aggregates that rapidly form in vitro in physiological conditions. These aggregates can be observed using SDS-PAGE, size-exclusion chromatography, dynamic light scattering, and real-time NMR analysis and are associated with decreased bioactivity as reflected by reduced drug-induced cellular proliferation and STAT3 phosphorylation. Furthermore, individual variability in the stability and detectability of pegfilgrastim in human sera is also observed. Pegfilgrastim levels display marked subject variability in sera from healthy donors incubated at 37 °C. The stability patterns of pegfilgrastim closely match the stability patterns of filgrastim, consistent with a key role for pegfilgrastim’s G-CSF moiety in driving formation of inactive aggregates. Taken together, our results indicate that individual variability and ELISA specificity for inactive aggregates are key factors to consider when designing and interpreting studies involving the measurement of serum pegfilgrastim concentrations.

The recombinant human granulocyte colony stimulating factor (G-CSF) product, filgrastim, and the related pegylated molecule, pegfilgrastim, have been licensed for the prevention of chemotherapy-induced neutropenia and its life-threatening complications. These drugs bind to cell surface G-CSF receptors (G-CSF-R) on mature neutrophils and their precursors, leading to increased cell division and expedited transit through the bone marrow to the peripheral blood and tissues1,2. Pegfilgrastim was developed by covalently linking a ~20kDa polyethylene glycol (PEG) molecule to the N-terminus methionine residue of filgrastim. PEGylation of G-CSF increases the total molecular weight from 18.8 to 39 kDa, while maintaining the biological activity of G-CSF3. However, the biological half-life of pegfilgrastim is significantly longer4, allowing administration once per chemotherapy cycle4–6. The difference in half-life between filgrastim and pegfilgrastim has been attributed to the presence of

1Office of Biotechnology Products, Office of Pharmaceutical Quality, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, Maryland, United States of America. 2Office of Clinical Pharmacology, Office of Translational Sciences, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, Maryland, United States of America. 3Office of Testing and Research, Office of Pharmaceutical Quality, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, Maryland, United States of America. 4Office of Biostatistics, Office of Translational Sciences, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, Maryland, United States of America. 5Office of Biostatistics, Office of Translational Sciences, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, Maryland, United States of America. *email: david.frucht@fda.hhs.gov
the PEG moiety on pegfilgrastim, which, by increasing the hydrodynamic size, decreases renal clearance\(^5\). In contrast to filgrastim, pegfilgrastim clearance is hypothesized to be mediated via the neutrophil G-CSF-R\(^1\).

U.S.-licensed Neulasta (pegfilgrastim) has a large commercial market ($4.7 B in 2017)\(^2\) and has recently been the target of biosimilar development in the United States, where three biosimilar products to US-licensed Neulasta have been FDA-approved to date. Pharmacokinetic (PK) similarity is an essential component of establishing a biosimilar product. The serum levels of pegfilgrastim following treatment, which are commonly determined using enzyme-linked immunosorbent assays (ELISAs), show a high degree of human subject variability\(^12\). One interpretation of the results of these studies is that the time-dependent decline in the levels of detected pegfilgrastim reflects clearance of the drug via G-CSF-R-mediated pathways, but this conclusion assumes that pegfilgrastim retains a stable ELISA signal in vivo.

We herein report that pegfilgrastim aggregates rapidly in physiological conditions, leading to reduced potency and ELISA detectability. Moreover, we observe that there is marked individual variability in the stability and detectability of pegfilgrastim in human sera. These factors should be considered in the design (PK study sample size, dosing, sample handling, etc.) and interpretation of clinical studies involving the measurement of pegfilgrastim concentrations.

**Results**

**Pegfilgrastim loses ELISA detectability in physiological conditions.** To monitor whether the detectability of pegfilgrastim is sustained in physiological conditions, pegfilgrastim was diluted at room temperature in a variety of commonly used physiological media or buffers. Following dilution, baseline pegfilgrastim levels determined using a commercial G-CSF ELISA (designated ELISA #1) were slightly lower compared to levels observed in samples of pegfilgrastim diluted in its recommended formulation buffer (10 mM acetate with 5% sorbitol and 0.004% polysorbate 20, pH 4.0\(^17\)), consistent with low surface binding to the microcentrifuge tubes used for studies. Pegfilgrastim levels showed a further time-dependent decline during incubation at 37 °C in some physiological conditions. As shown in Fig. 1b, the detection of pegfilgrastim in DMEM or Ringer’s solution declined approximately 70% from baseline, and a smaller, but statistically significant decline was observed in Hanks Balanced Salt Solution (HBSS). Downward trends were also observed when pegfilgrastim was diluted in PBS or Normal Saline, but the differences were not statistically significant (p = 0.08). Levels remained constant in the manufacturer’s non-physiological formulation buffer.

To determine whether the decline in detectability observed in certain physiological buffer conditions would also be observed in human sera, pegfilgrastim was spiked into sera from 10 individuals (Donors S01-S10) and incubated at 37 °C and monitored by ELISA in a time course experiment. The average levels detected in these samples following 12h of incubation was ~1/2 that of baseline levels (T = 0; Fig. 1c). As observed in certain balanced salt solutions and media that mimic physiological conditions, there was a time-dependent decline of pegfilgrastim detected using ELISA #1 when the drug was incubated in human sera. By the conclusion of the 4-day experiment, the average detected levels had decreased to <1/3 of baseline, however substantial subject variability was observed, with one outlying serum maintaining ~75% of baseline pegfilgrastim levels.

**Pegfilgrastim forms aggregates in physiological conditions.** Because a loss of ELISA detectability could be due to structural changes of pegfilgrastim in physiological conditions, as have been previously described at physiological pH\(^17,18\), the structural integrity of pegfilgrastim was investigated using a series of orthogonal techniques. Proteolytic fragments were not observed during SDS-PAGE analysis performed under reducing conditions (pre-treatment with 50 mM dithiothreitol at 70 °C for 10 minutes), which would indicate that pegfilgrastim does not undergo proteolysis when incubated in DMEM (Fig. 2a). However, when analysed by non-reducing SDS-PAGE, higher molecular weight bands were detected, whose levels increased in a time-dependent manner (Fig. 2b). These findings suggest that covalently-linked disulphide bonds might be involved in the aggregation of pegfilgrastim.

Pegfilgrastim aggregate formation in physiological conditions (DMEM, 37 °C) was also detected by high throughput dynamic light scattering (HT-DLS), which revealed the rapid accumulation of ~40 nm size pegfilgrastim complexes (Fig. 2c). In contrast, the molecular size remained ~10 nm when maintained in formulation buffer. Size exclusion chromatography (SEC) revealed similar increases in aggregate content and depletion of monomer content in DMEM (Fig. 2d). As with results obtained using HT-DLS, SEC analysis showed that the majority of pegfilgrastim aggregates following 24 h in this physiological condition.

Various chemical mechanisms, such as deamidation, reduction, oxidation, and hydrolysis, may be involved in the structural stability of the pegfilgrastim protein moiety\(^19\). This G-CSF region comprises 175 amino acid residues containing two native disulphide bonds at positions 36–42 and 64–74 and one free cysteine at position 17, which is partially solvent-exposed\(^20\). The potential involvement of the Cys 17-associated disulphide bond in the aggregation of G-CSF has been of special interest in the field\(^21\). Previous studies report that G-CSF is prone to form dimeric aggregates under physiological conditions (37 °C, pH 7.0), and aggregated G-CSF contains a population that is disulphide cross-linked\(^13,22\). Pegylation of G-CSF has a substantial protective effect on its stability\(^18\), so it was unclear whether pegfilgrastim would share similar characteristics.

We therefore assessed the roles of pH and disulphide cross-linking during the aggregation of pegfilgrastim. To this end, we determined the concentrations of free thiol levels of pegfilgrastim before and after incubation in DMEM that were titrated to pH 4, 7, or 8. As shown in Fig. 2e, free thiol levels were very similar immediately following reconstitution in these media. These results suggest that a rapid, pH-dependent transition involving disulphide shuffling is unlikely. However, following 15 hours of incubation at 37 °C, there was a substantial loss in free thiol levels at pH 7 and pH 8, but not pH 4. This can likely be explained by previously described conformational changes that allow increased accessibility of the free Cys 17\(^20\) at higher pH levels.
We next used 1D 1H Nuclear Magnetic Resonance (NMR) to obtain more detailed information regarding the structure of the pegfilgrastim aggregates that form in physiological conditions. In contrast to the stable peak intensities observed for pegfilgrastim maintained in formulation buffer (Figs. 3a,b and S1) or PBS (Fig. 3c,d), NMR studies revealed a time-dependent decrease in the protein peak intensity in DMEM (Figs. 3e and S2). As this drop in the protein peak intensity was accompanied by a stable PEG signal (Fig. 3f), our findings support a model in which the filgrastim moiety of pegfilgrastim, and not the PEG chain, drives aggregation in DMEM. Moreover, the sharp PEG-associated NMR peak suggests that PEG remains highly flexible on the pegfilgrastim surface (Figs. 3f and S1), or water exposed, consistent with a previous study that reported the formation of soluble pegfilgrastim aggregates under physiological conditions18. Collectively, these orthogonal assays demonstrate that pegfilgrastim rapidly aggregates in certain physiological conditions, forming soluble particles that initially plateau in size around 40 nm (Fig. 2c). Subsequent structural changes then continue to occur that correspond with further decreased ELISA detectability (Figs. 2b,d,e, 3e, S2b,c).

Pegfilgrastim detectability in serum stability studies is ELISA-dependent. We considered the possibility that commercial ELISAs have varying sensitivities to pegfilgrastim aggregates that form in physiological conditions. To address this question experimentally, pegfilgrastim was spiked into the sera from 15 individuals (Donors S01-S15), and levels at baseline and following 24 h of incubation at 37 °C were then determined using three different commercial ELISAs detecting the G-CSF (ELISAs #1 and #2) or PEG (ELISA #3) regions of pegfilgrastim. As shown in Fig. 4a, the various ELISA formats provided strikingly different results in two respects. First, the determined concentrations varied markedly even at baseline, but this was not unexpected given their assay-specific concentration standards. Secondly, and more importantly, there were differences in the relative
degrees of sample stability detected at 37 °C. Levels determined using commercial G-CSF ELISA #1 assay showed a very large decline following incubation in physiological conditions. A commercial PEG ELISA detection kit (3) showed a statistically significant drop in detection as well, albeit only a ~20% drop on average. In contrast, commercial G-CSF ELISA #2 showed no statistically significant decrease in detected levels, confirming that commercial ELISAs have differing capacities to distinguish monomeric pegfilgrastim from the aggregates that form in physiological conditions.

We next investigated whether the loss of detectability using ELISA #1 was associated with the filgrastim component of pegfilgrastim. Filgrastim was spiked into individual sera (n = 15, Donors S16–S30) and incubated at 37 °C (Fig. 4a). In parallel with the pegfilgrastim results (Fig. 4a), the mean levels of filgrastim detected following

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**Figure 2.** Determinations of the structure of pegfilgrastim in DMEM using orthogonal methods. (a,b) Pegfilgrastim was reconstituted in formulation buffer (Contl) or DMEM, incubated at 37 °C for the various time points indicated, and analysed by SDS-PAGE in (a) reducing or (b) non-reducing conditions. (c) The hydrodynamic sizes of pegfilgrastim in DMEM or formulation buffer (F.B.) at 37 °C were monitored continuously for 24 h using high throughput dynamic light scattering. (d) Pegfilgrastim was diluted in DMEM and incubated at 37 °C. Aggregation was assessed using SEC-HPLC at the indicated time points. (e) Pegfilgrastim was diluted to 400 µg/mL with DMEM that pH has previously been titrated to pH 4, pH 7 or pH 8, and the samples were incubated at 37 °C for 1.5 h. Free thiol levels were measured as shown (n = 4, **p < 0.001 Student’s T-test).
24 h of incubation in sera were much lower compared to baseline levels when ELISA #1 was used for measurements (~35% baseline levels). In contrast, only a modest reduction was observed when ELISA #2 was used (~75% baseline levels). These results paralleled those of pegfilgrastim and are consistent with a model in which the structural changes involving the G-CSF moiety underlies the reduced ELISA#1 detectability of pegfilgrastim in physiological conditions. PEGylation may serve to slow this degradative process, as has been previously proposed

Figure 3. Real-time 1D $^1$H NMR spectra for pegfilgrastim at 37°C in different buffers (a,b) formulation, (c,d) 0.5× PBS, (e,f) DMEM. Shown are the overlays of three 1D $^1$H NMR spectra for pegfilgrastim amide region peaks (a,c,e) or PEG peak (b,d and f) in these conditions at 1 h (red), 20 h (green) and 43 h (blue).
The stability of pegfilgrastim is highly variable among individuals. Human donor variability (Fig. 5a), was examined further in stability experiments using individual sera from Donors S16-S25. Drug levels at baseline and following 24 h incubation at 37 °C were measured using ELISA #1 in a total of four independent
sample preparations. As is evident in Fig. 5a, there was a marked reduction in detected levels of pegfilgrastim, which was highly donor-dependent. A parallel experiment (Fig. 5b) was performed in which filgrastim (200 ng/mL) was spiked into the same 10 individual sera (S16-25), and the levels of filgrastim at baseline and following 24 h treatment at 37 °C were measured using ELISA #1. Interestingly, the relative stability pattern of pegfilgrastim among the tested individuals closely matched that of filgrastim (Fig. 5a,b). As shown in Fig. 5c, there was a direct correlation between the serum stability of pegfilgrastim and the serum stability of filgrastim (r² = 0.93), supporting the conclusion that the G-CSF moiety is central to the degradation of pegfilgrastim in human serum.

Pegfilgrastim bioactivity correlates directly with ELISA detectability. It was also critical to investigate the correlation between the decreased ELISA detectability of pegfilgrastim in physiological conditions and its biological activity to understand the potential implications of these results. To this end, we preincubated pegfilgrastim in DMEM in low protein binding microcentrifuge tubes for varying lengths of time (0–48 h) at 37 °C, which led to a time-dependent decrease in ELISA#1 detectability (Fig. 6a, lower panel). The bioactivity of pegfilgrastim in these samples was then assessed by analysing the capacity of these samples to stimulate the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in NFS-60 cells, a G-CSF receptor-expressing murine cell line derived from a myelogenous leukaemia. Phosphorylation of tyrosine Y705 of STAT3 is the primary consequence of G-CSF-R activation in neutrophil and myeloid lineages25,26. The capacity of the pegfilgrastim samples to induce STAT3 phosphorylation decreased with their time of pre-incubation in physiological conditions, paralleling the drop of pegfilgrastim ELISA#1 detectability (Fig. 6a, upper and lower panels).

Next, we examined the correlation of ELISA detectability and bioactivity of pegfilgrastim in serum. Two donors were identified, S08 and S10, whose pegfilgrastim serum stability characteristics differed greatly. Serum from donors S08 and S10 was spiked with 400 ng/mL of pegfilgrastim and incubated at 37 °C. Following 24 h in these conditions, the pegfilgrastim level in Donor S08 serum was ~70% baseline, whereas a <10% baseline level was observed in serum from Donor S10 (Fig. 1c). The pY-STAT3 level stimulated by the S10 sample preincubated for 24 h was markedly lower than that of the corresponding S08 sample, indicating reduced bioactivity in this sample. In contrast, the S08 sample preincubated for 24 h showed no substantial reduction in pY-STAT3 levels, correlating with its retained ELISA detectability in physiological conditions.

In addition, the pegfilgrastim samples incubated in S08 and S10 sera were assessed for their capacity to induce proliferation of NFS-60 cells27–29, which are widely used in bioassays for assessing bioactivity of pegfilgrastim and filgrastim. Paralleling STAT3 activation results; the proliferative activity of pegfilgrastim incubated in S10 serum for 24 h was markedly lower than that of corresponding samples incubated in S08 serum (Fig. 6c). Taken together, pegfilgrastim bioactivity, as determined by signal transduction capacity and downstream induction of proliferation, correlated directly with detectability using a commercial ELISA with differential specificity for non-aggregated vs. aggregated pegfilgrastim.
Discussion

Our results support the conclusion that pegfilgrastim aggregates in certain physiological conditions. This dynamic aggregation process is mediated by physicochemical changes associated with the filgrastim moiety and ultimately leads to irreversible disulfide bond formation. Whether surface binding to microcentrifuge containers accelerates this process is unclear, but the use of low protein binding surfaces in our experiments minimized this factor. The detectability of pegfilgrastim aggregates varies between several commercial G-CSF and PEG ELISA detection kits, at least one of which has served as a development platform for assays for clinical pegfilgrastim concentration determinations\textsuperscript{12–14}. Moreover, the formation of inactive pegfilgrastim aggregates that are either directly (physiological buffers) or indirectly (human sera) observed occurs at physiologically relevant concentrations, suggesting these aggregates may impact the interpretation of pegfilgrastim ELISA results \textit{in vitro} or \textit{ex vivo} in clinical PK studies.

The pH of the environment is likely one component underlying the physicochemical basis for the aggregation of filgrastim and pegfilgrastim in physiological conditions. At low pH, filgrastim, a member of the four-helix bundle class of cytokines, is compact, shows a high degree of $\alpha$-helicity, and is resistant to aggregation\textsuperscript{22,30}. However, spectroscopic analysis of filgrastim aggregates reveals that distinct conformational changes occur between pH 5 and pH 7.0\textsuperscript{22,30,31}, consistent with the change in size of pegfilgrastim that we observed by HT-DLS immediately upon addition to DMEM. Previous published reports indicate that filgrastim aggregates and rapidly precipitates at pH 6.9 and 37 °C\textsuperscript{24}. However, these initial studies investigating formation of filgrastim aggregates were performed at high concentrations that are not physiologically relevant. Filgrastim aggregate formation was concentration-dependent, and, thus, it was thought that aggregation would not be a concern at the picomolar range concentrations found in the bloodstream\textsuperscript{18,22}.

Pegfilgrastim has been proposed to form aggregates via the same degradation pathway as filgrastim. Both have been shown to have almost identical secondary structural transitions, accompanied by the formation of aggregates with nearly identical covalent characteristics\textsuperscript{18}. Our finding of highly comparable patterns of human subject variability of filgrastim and pegfilgrastim stability in individual sera further supports a common aggregation pathway for these two molecules. Pegfilgrastim incorporates a PEG moiety, known to increase the solubility and stability of proteins\textsuperscript{32}, likely allowing the formation of soluble aggregates under the same conditions that cause G-CSF to precipitate\textsuperscript{22,24}. It is also conceivable that the conjugation of PEG to filgrastim decreases its rate
of aggregation. Importantly, it should be considered that, in contrast to filgrastim, pegfilgrastim has a prolonged circulating half-life, increasing the likelihood that aggregation may impact its detectability prior to clearance (e.g., by receptor-mediated pathways).

We hypothesize that formation of pegfilgrastim aggregates masks certain epitopes recognized by commercial ELISAs. This would be consistent with the high level of aggregate detection with a commercial PEG ELISA detection kit, as NMR studies indicate that the hydrophilic PEG components in pegfilgrastim aggregates remain directed outward toward the water interface. In this scenario, hidden epitopes would be in unexposed regions in the G-CSF components of the pegfilgrastim aggregates. However, proprietary information regarding the epitopes recognized by the antibodies used in commercial ELISAs is not available to confirm this directly. We are currently conducting studies to evaluate the performance of two of the ELISA assays described in this study according to FDAs Bioanalytical Method Validation Guidance33.

Based on our findings in certain physiological buffers, the conversion of pegfilgrastim in human sera into inactive aggregates was expected. Nevertheless, the level of individual variability in the stability of pegfilgrastim in human sera samples was striking. In this regard, pegfilgrastim has been shown to display considerable subject variability in human PK studies12–16. Previously this variability was attributed to variability in cellular uptake, but our data suggest that differences in serum stability may play a large role. Subject variability in the serum stability of pegfilgrastim could represent a confounding factor in the PK studies used to establish PK similarity, supporting advantages for crossover study designs that mitigate this concern.

Although our results elucidate some of the factors that impact the potency and detectability of pegfilgrastim in physiological conditions, future studies are indicated to address remaining questions. For example, the primary mechanism of pegfilgrastim clearance from the circulation has been thought to be neutrophil-mediated clearance via G-CSF-R. This receptor binds pegfilgrastim, and the drug-receptor complex is internalized and degraded inside the cell34. Although ELISA results involving serum samples are similar to those with physiological media, we have not directly confirmed that pegfilgrastim forms aggregates in vivo. It will be valuable to develop methods to detect and quantify pegfilgrastim aggregates in vivo and, if present, investigate their impact on pegfilgrastim bioactivity and detectability. In addition, the human factors in serum that affect pegfilgrastim stability remain to be identified. It will also be important to determine whether certain product variants present in production lots, or the deliberate targeting of quality attributes (e.g., glycosylating the protein), could alter serum stability characteristics. Nevertheless, the data described herein are sufficient to warrant caution regarding the limitations of the interpretation of pegfilgrastim concentrations derived from ELISA-based methods, which have different capacities to differentiate non-aggregated vs. aggregated and biologically active vs. inactive products. Moreover, the impact of individual variability on the stability of protein therapeutics such as pegfilgrastim, highlight the need for a robust PK study design to meet endpoints for biosimilar development.

Methods

Human serum samples used in this study were acquired from a commercial source (Equitech Enterprises, Inc., Kerrville, Texas), and personal identifying information was not available, so none of the studies was considered human subject research after consultation with a representative from CDER's institutional review board. The anonymized serum samples were collected by the commercial source from normal adult individuals, with a diverse range of ethnicity and ages of both genders. The serum aliquots were stored at −70 °C and thawed on ice before being used in experiments.

ELISA assays for pegfilgrastim and filgrastim level measurements. Three commercially sourced enzyme-linked immunosorbent assay (ELISA) kits were used to quantitatively assess levels of pegfilgrastim and filgrastim in sera, media, or physiological balanced salt buffers as follows: (1) ELISA #1 (Human G-CSF Quantikine ELISA Kit, Cat. DC550, R&D Systems); (2) ELISA #2 (G-CSF Human Instant ELISA™ Kit, BMS2001INST, Invitrogen); and (3) ELISA #3 (PEGylated protein ELISA kit, ADI900-213-0001, Enzo). The assays were performed following the manufacturers’ recommended protocols, modified as indicated. To minimize the interference of surface binding effects, Eppendorf LoBind microcentrifuge tubes (Eppendorf®, # 022431081) were used in experiments involving incubation of low concentrations of pegfilgrastim in protein-free buffers/DMEM. The three commercial ELISA assays were not independently validated as per the FDA’s Bioanalytical Method Validation Guidance31. The rh-G-CSF standards provided with the Kits #1 and #2 were used as calibrators for relative quantitation. Serial dilutions of Neulasta (pegfilgrastim) using the dilution buffer provided with kit #3 were used for relative quantitation in the anti-PEG ELISA assay. The optical densities of ELISA plate wells were acquired using a Molecular Device SpectraMax Plus 384 Micro plate reader, and a four-parameter logistic (4-PL) curve-fit was applied for calculating the results.

NFS-60 cells proliferation and STAT3 activation. Determinations of STAT3 activation were used to assess the bioactivity of the pegfilgrastim samples. m-NFS-60 cells (ATCC®-1838™) at a concentration of 5–7 × 10^5 cell/mL, were stimulated for 30 min with samples containing pegfilgrastim pre-incubated in DMEM or human sera (1 ng/mL final concentration). Total protein cell lysates were prepared using RAPA buffer supplied with Halt™ proteases and phosphatases inhibitors (Thermo #1861280). The protein bands were separated and revealed by standard SDS-PAGE gel and Western Blot technologies. Antibodies used in Western Blot detection were rabbit anti-GCSF (Novus Biologicals, NBP1-89894), mouse anti-STAT3 (pY705) (BD 612357), and STAT3 mAb (9D8) (MA1-13042). The bioactivity of the pegfilgrastim in samples was also assessed using a m-NFS-60 cell proliferation assay; NSF-60 cells (2–3 × 10^4/mL) grown in complete medium were stimulated by the addition of test samples, followed by continued culture for 48 hours. Cellular proliferation was quantitatively assessed using the WST-1 Colorimetric Assay (Sigma Cat# 11-644-807-001), performed per the manufacturer recommended protocol.
Reduced and non-reduced SDS-PAGE analysis electrophoresis. Pegfilgrastim was reconstituted in formulation buffer or DMEM to a concentration of 5 μg/mL and maintained at 37 °C for the indicated time periods (up to 68 h). SDS-PAGE analysis was performed using standard techniques and the NuPAGE 4–12% Bis-Tris Gel system (Invitrogen) following the manufacturer suggested protocol. A commercial silver stain assay (Pierce, Cat. 24612) was used to reveal protein bands on the gel.

SEC-HPLC. To assess changes in quaternary structure, pegfilgrastim was analysed by size exclusion chromatography (SEC) using an Agilent AdvanceBio SEC. 130 Å, 7.8 × 300 mm, 2.7 μm column (cat# PL1180-5350) and 50 mm guard (cat# PL1180-1350) on an Agilent 1260 HPLC. The mobile phase, flow rate, and run time were the same as previously described27. Pegfilgrastim (Amgen, Lot# 1063064,) was diluted in formulation buffer according to the package insert to a concentration of 200 μg/mL. Samples were further diluted 1:40 to a final concentration of 5 μg/mL into phenol red-free DMEM (ThermoFisher, cat #31053-028) in a low protein binding microcentrifuge tube (Eppendorf LoBind) and incubated at 37 °C for 0–24 h prior to transfer to an HPLC vial for analysis. SEC-HPLC samples were injected at 5 μg/mL with a fixed injection volume of 100 μL, and the protein was detected by measuring UV absorbance at 214 nm.

High throughput dynamic light scattering. A DynaPro II Plate Reader HT-DLS instrument from Wyatt Technologies (Santa Barbara CA) was used to evaluate the hydrodynamic size of pegfilgrastim drug product in DMEM. An algorithm-driven high throughput screening method was developed to monitor the size changes of pegfilgrastim maintained in formulation buffer or DMEM at a concentration of 1.25 mg/mL and monitored continuously under physiological temperature conditions35. DYNAMICS software from the same vendor was used to record and analyse the light scattering data to determine the hydrodynamic size of the pegfilgrastim in solution.

Quantitation of free thiol levels. Pegfilgrastim was diluted in DMEM to a final concentration of 400 μg/mL and incubated at 37 °C for the indicated times in Eppendorf LoBind microcentrifuge tubes. The levels of free thiol of pegfilgrastim samples were measured using the commercial Measure-iT Thiol Assay Kit (Molecular Probes, M30550), following the manufacturer’s recommended protocol.

NMR spectroscopy. To evaluate the structure of pegfilgrastim in formulation buffer, PBS buffer, or DMEM medium, spectra of 1D 1H were collected on a Bruker Avance III 850 MHz spectrometer equipped with a QCI cryo-probe. The NMR samples that were analyzed comprised 0.035 mL of pegfilgrastim (Neulasta®), 0.02 mL of D2O, and 0.295 mL of formulation buffer, PBS buffer, or DMEM medium. The experimental temperature was 37 °C. The 1D 1H NMR experiments were performed using Bruker pulse program p39199g with 128 scans averaged. The experimental time for 1D 1H NMR was 6 minutes. Spectra were continuously collected over a time course of 48 h to monitor structural changes. NMR data were processed and analyzed using MestReNova 11.0 software (Mestrelab Research S.L.).

Statistics. Student’s T-test and linear regression analyses were performed using GraphPad Prism 6 software. The SAS procedure Proc Mixed analysis was applied to determine the subject effect on pegfilgrastim and filgrastim stability in experiments shown in Fig. 5a,b. As illustrated by Eq. (1), $y_{ij}$ is the ratio of the pegfilgrastim level measured by ELISA at 24 h over the pegfilgrastim level at the baseline for the $i$th subject and $j$th sample, $y_{ij}$ is the pegfilgrastim level at the baseline for the $i$th subject and $j$th sample, $a_i$ is the $i$th subject and is a random effect, and $e_{ij}$ is the random error term for the $i$th subject and $j$th sample. In the mixed effect model (1), we further assumed that $a_i$ is a normal variable with a mean of zero and a variance of $\sigma_a^2$, $e_{ij}$ is a normal variable with a mean of zero and a variance of $\sigma_e^2$, and $a_i$ is independent of $e_{ij}$.

$$y_{ij} = \mu + \beta_i y_{ij0} + a_i + e_{ij} \quad (1)$$

Here $I = 1, 2, \ldots, 10$, and $j = 1, 2, 3, 4$.

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References
1. Roberts, A. W. G-CSF: a key regulator of neutrophil production, but that’s not all! Growth Factors 23, 33–41, https://doi.org/10.1080/0897190500553836 (2005).
2. Fukunaga, R., Seto, Y., Mizushima, S. & Nagata, S. Three different mRNAs encoding human granulocyte colony-stimulating factor receptor. Proc. Natl Acad. Sci. USA 87, 8702–8706 (1990).
3. Molinex, G. The design and development of pegfilgrastim (PEG-metHuG-CSF, Neulasta). Curr. Pharm. Des. 10, 1235–1244 (2004).
4. Yang, B. B. & Kido, A. Pharmacokinetics and pharmacodynamics of pegfilgrastim. Clin. Pharmacokinet. 50, 295–306, https://doi.org/10.2165/11586040-000000000-00000 (2011).
5. Lyman, G. H. Pegfilgrastim: a granulocyte colony-stimulating factor with sustained duration of action. Expert. Opin. Biol. Ther. 5, 1635–1646, https://doi.org/10.1517/14712998.5.12.1635 (2005).
6. Mehta, H. M., Malandra, M. & Corey, S. J. G-CSF and GM-CSF in Neutropenia. J. Clin. Pharm. 37, 1341–1349, https://doi.org/10.1049/jipha.1500861 (2015).
7. Yang, B. B., Lum, P. K., Hayashi, M. M. & Roskos, L. K. Polyethylene glycol modification of filgrastim results in decreased renal clearance of the protein in rats. J. Pharm. Sci. 93, 1367–1373, https://doi.org/10.1002/jps.20024 (2004).
8. Roskos, L. K., Lum, P., Lockbaum, P., Schwab, G. & Yang, B. B. Pharmacokinetic/pharmacodynamic modeling of pegfilgrastim in healthy subjects. J. Clin. Pharmacology 46, 747–757, https://doi.org/10.1177/0022323906288731 (2006).
9. Yang, B. B., Kido, A., Salfi, M., Swan, S. & Sullivan, J. T. Pharmacokinetics and pharmacodynamics of pegfilgrastim in subjects with various degrees of renal function. J. Clin. Pharmacology 48, 1025–1031, https://doi.org/10.1177/0022323906288731 (2008).
10. Layton, J. E., Hockman, H., Sheridan, W. P. & Morstyn, G. Evidence for a novel in vivo control mechanism of granulopoiesis: mature cell-related control of a regulatory growth factor. Blood 74, 1303–1307 (1989).
11. EvaluatePharma. Phama World Preview 2017, Outlook to 2022. 10th edn. (2017).
12. Yamamoto, N. et al. A pharmacokinetic and dose escalation study of pegfilgrastim (KRN125) in lung cancer patients with chemotherapy-induced neutropenia. Jpn. J. Clin. Oncol. 39, 425–430, https://doi.org/10.1093/jjco/hyp038 (2009).
13. Fox, E. et al. Randomized trial and pharmacokinetic study of pegfilgrastim versus filgrastim after dose-intensive chemotherapy in young adults and children with sarcomas. Clin. Cancer Res. 15, 7761–7767, https://doi.org/10.1158/1078-0432.CCR-09-0761 (2009).
14. Nakov, R. et al. In Br. J. Clin. Pharmacol. 84, 2790–2801 (2018).
15. Zamboni, W. C. Pharmacokinetics of pegfilgrastim. Pharmacotherapy 23, 95–145 (2003).
16. Yang, B. B., Morrow, P. K., Wu, X., Moxness, M. & Padhi, D. Comparison of pharmacokinetics and safety of pegfilgrastim administered by two delivery methods: on-body injector and manual injection with a prefilled syringe. Cancer Chemother. Pharmacol. 75, 1199–1206, https://doi.org/10.1007/s00280-015-2731-x (2015).
17. Piedmonte, D. M. & Treuheit, M. J. Formulation of Neulasta (pegfilgrastim). Adv. Drug. Deliv. Rev. 60, 50–58, https://doi.org/10.1016/j.addr.2007.04.017 (2008).
18. Rajan, R. S. et al. Modulation of protein aggregation by polyethylene glycol conjugation: GCSF as a case study. Protein Sci. 15, 1063–1075, https://doi.org/10.1002/pro.170 (2006).
19. Reubsaet, J. L. et al. Oxidation of recombinant methionyl human granulocyte colony stimulating factor. J. Pharm. Biomed. Anal. 17, 283–289, https://doi.org/10.1016/S0731-7085(97)00199-4 (1998).
20. Arakawa, T., Prestrehlski, S. J., Narhi, L. O., Boone, T. C. & Kenney, W. C. Cysteine 17 of recombinant human granulocyte-colony stimulating factor is partially solvent-exposed. J. Protein Chem. 12, 525–531, https://doi.org/10.1007/BF01023317 (1993).
21. Torosantucci, R., Schonheit, C. & Jiskoot, W. Oxidation of therapeutic proteins and peptides: structural and biological consequences. Pharm. Res. 31, 541–553, https://doi.org/10.1007/s11095-013-1199-9 (2014).
22. Raso, S. W. et al. Aggregation of granulocyte-colony stimulating factor in vitro involves a conformationally altered monomeric state. Protein Sci. 14, 2246–2257, https://doi.org/10.1111/j.110105180405 (2005).
23. Chi, E. Y. et al. Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte-colony-stimulating factor. Protein Sci. 12, 903–913, https://doi.org/10.1101/pdb.0235703 (2003).
24. Krishnan, S. et al. Aggregation of granulocyte colony stimulating factor under physiological conditions: characterization and thermodynamic inhibition. Biochem. 41, 6422–6431 (2002).
25. McMenemy, M. L. et al. STAT-3 activation is required for normal G-CSF-dependent proliferation and granulocytic differentiation. Immun. 14, 193–204 (2001).
26. Huang, G., Yan, H., Ye, S., Tong, C. & Ying, Q. L. STAT3 phosphorylation at tyrosine 705 and serine 727 differentially regulates mouse ESC fates. Stem Cell 32, 1149–1160, https://doi.org/10.1002/stem.1609 (2014).
27. Broxk, S., Scrochi, L., Shah, N. & Dowd, J. A demonstration of analytical similarity comparing a proposed biosimilar pegfilgrastim and reference pegfilgrastim. Biol. 48, 28–38, https://doi.org/10.1016/j.biosimilars.2017.06.001 (2017).
28. EuropenPharmacopoeia. Filgrastim concentrated solution. Vol. 6.3 (01/2009:2206).
29. Tiwari, K. et al. A sensitive WST-8-based bioassay for PEGylated granulocyte colony stimulating factor using the NFS-60 cell line. Pharm. Biol. 53, 849–854, https://doi.org/10.3109/13880209.2014.943248 (2015).
30. Kolvenbach, C. G. et al. Granulocyte-colony stimulating factor maintains a thermally stable, compact, partially folded structure at pH2. J. Pept. Res. 50, 310–318 (1997).
31. Narhi, L. O., Kenney, W. C. & Arakawa, T. Conformational changes of recombinant human granulocyte-colony stimulating factor induced by pH and guanidine hydrochloride. J. Protein Chem. 10, 359–367 (1991).
32. Palt, T., Esladiary, R. & Gandhi, R. The effect of PEGylation on the stability of small therapeutic proteins. Pharm. Dev. Technol. 16, 441–448, https://doi.org/10.3109/10837450.2010.558330 (2011).
33. U.S. Food and Drug Administration Center for Drugs Evaluation and Reseach. Guidance for Industry: Bioanalytical Method Validation, https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf (May 2018).
34. Robinson, C. R. Reduce, reuse, and recycle. Nat. Biotechnol. 20, 879–880, https://doi.org/10.1038/nbt0902-879 (2002).
35. Bhinder, A. A., Chiang, M. J., Venna, R., Beaucage, S. & Brorson, K. High-Throughput In-Use and Stress Size Stability Screening of Protein Therapeutics Using Algorithm-Driven Dynamic Light Scattering. J. Pharm. Sci. 107, 2055–2062, https://doi.org/10.1016/j.xphs.2018.04.017 (2018).

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Author contributions
TX. and D.F. wrote the main manuscript text; T.X., H.F., D.F., P.A., M.C., A.B., F.S., A.S., S.P., K.C., C.A., R.D., K.B., K.H. and S.R. performed or supervised experiments; and S.S., W.O. and P.L. provided technical expertise and advice. M.S. provided expertise regarding the statistical analysis. All authors reviewed and/or edited the manuscript.

Competing interests
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