

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 May 2012 (18.05.2012)

PCT

(10) International Publication Number
WO 2012/064845 A2

(51) International Patent Classification:
C07K 14/535 (2006.01)

(21) International Application Number:
PCT/US2011/059976

(22) International Filing Date:
9 November 2011 (09.11.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/411,826 9 November 2010 (09.11.2010) US
61/530,812 2 September 2011 (02.09.2011) US

(71) Applicant (for all designated States except US): **NEKTAR THERAPEUTICS** [US/US]; 455 Mission Bay Boulevard South, Suite 100, San Francisco, CA 94158 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BOSSARD, Mary, J.** [US/US]; 120 Kelvingrove Drive, Madison, AL 35758 (US). **ZHANG, Ping** [US/US]; 102 Rainbow Glen Circle, Madison, AL 35758 (US).

(74) Agents: **WILSON, Mark, A.** et al.; Nektar Therapeutics, 455 Mission Bay Boulevard South, Suite 100, San Francisco, CA 94158 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: G-CSF POLYMER CONJUGATES HAVING A RELEASABLE LINKAGE

(57) Abstract: The present invention provides G-CSF-polymer conjugates having a releasable linkage. Methods of making conjugates and methods for administering conjugates are also provided.



WO 2012/064845 A2

G-CSF POLYMER CONJUGATES HAVING A RELEASABLE LINKAGE

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority of Provisional Patent Application No. 61/411,826 filed November 9, 2010 and Provisional Patent Application No. 61/530,812 filed September 02, 2011, the contents both of which are expressly incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

[0002] The present invention relates generally to water-soluble polymer-G-CSF moiety conjugates having a releasable linkage to thereby release the G-CSF moiety *in vivo*. In addition, the invention relates to, among other things, methods for synthesizing such conjugates, methods for purifying the conjugates, compositions comprising the conjugates, methods for administering the conjugates, and so on.

BACKGROUND

[0003] Scientists and clinicians face a number of challenges in their attempts to develop active agents into forms suited for delivery to a patient. Active agents that are polypeptides, for example, are often delivered via injection rather than orally. In this way, the polypeptide is introduced into the systemic circulation without exposure to the proteolytic environment of the stomach. Injection of polypeptides, however, has several drawbacks. For example, many polypeptides have a relatively short half-life, thereby necessitating repeated injections, which are often inconvenient and painful. Moreover, some polypeptides can elicit one or more immune responses with the consequence that the patient's immune system attempts to destroy or otherwise neutralize the immunogenic polypeptide. Of course, once the polypeptide has been destroyed or otherwise neutralized, the polypeptide cannot exert its intended pharmacodynamic activity. Thus, delivery of active agents such as polypeptides is often problematic even when these agents are administered by injection.

[0004] Some success has been achieved in addressing the problems of delivering active agents via injection. For example, conjugating the active agent to a water-soluble polymer has resulted in polymer-active agent conjugates having reduced immunogenicity and antigenicity. In addition, these polymer-active agent conjugates often have greatly increased half-lives compared

to their unconjugated counterparts as a result of decreased clearance through the kidney and/or decreased enzymatic degradation in the systemic circulation. As a result of having a greater half-life, the polymer-active agent conjugate requires less frequent dosing, which in turn reduces the overall number of painful injections and inconvenient visits with a health care professional. Moreover, active agents that were only marginally soluble demonstrate a significant increase in water solubility when conjugated to a water-soluble polymer.

[0005] Due to its documented safety as well as its approval by the FDA for both topical and internal use, polyethylene glycol has been conjugated to a number of active agents. When an active agent is conjugated to a polymer of polyethylene glycol or "PEG," the conjugated active agent is conventionally referred to as "PEGylated." The commercial success of PEGylated active agents such as PEGASYS[®] PEGylated interferon alpha-2a (Hoffmann-La Roche, Nutley, NJ), PEG-INTRON[®] PEGylated interferon alpha-2b (Schering Corp., Kenilworth, NJ), and NEULASTA[®] PEG-filgrastim (Amgen Inc., Thousand Oaks, CA) demonstrates that administration of a conjugated form of an active agent can, but will not necessarily, have significant advantages over the unconjugated counterpart. Small molecules such as distearoylphosphatidylethanolamine (Zalipsky (1993) *Bioconjug. Chem.* 4(4):296-299) and fluorouracil (Ouchi *et al.* (1992) *Drug Des. Discov.* 9(1):93-105) have also been PEGylated. Harris *et al.* have provided a review of the effects of PEGylation on pharmaceuticals. Harris *et al.* (2003) *Nat. Rev. Drug Discov.* 2(3):214-221.

[0006] Despite these successes, conjugation of a polymer to an active agent to provide a commercially relevant drug is often challenging. For example, conjugation can result in the polymer being attached at or near a site on the active agent that is necessary for pharmacologic activity (e.g., at or near a binding site). Such conjugates may therefore have unacceptably low activity due to, for example, the steric effects introduced by the polymer. Attempts to remedy conjugates having unacceptably low activity can be frustrated when the active agent has few or no other sites suited for attachment to a polymer. Thus, additional PEGylation alternatives have been desired.

[0007] One suggested approach for solving this and other problems is "reversible PEGylation" where the unmodified active agent (or a moiety having increased activity compared to the PEGylated active agent) is released. For example, reversible PEGylation has been disclosed in the field of cancer chemotherapies. See Greenwald (1997) *Exp. Opin. Ther. Patents* 7(6):601-609. U.S. Patent Application Publication No. 2005/0079155 describes conjugates using

reversible linkages. As described in the foregoing publication, reversible linkages can be effected through the use of an enzyme substrate moiety. It has been pointed out, however, that approaches relying on enzymatic activity are dependent on the availability of enzymes. See Peleg-Schulman (2004) *J. Med. Chem.* 47:4897-4904. Patient variability around the amount and activity of these enzymes can introduce inconsistent performance of the conjugate among different populations. Thus, additional approaches that do not rely on enzymatic processes for polymer release are desirable.

[0008] Another approach for reversible PEGylation is described in U.S. Patent No. 7,060,259, which describes (among other things) water-soluble prodrugs in which a biologically active agent is linked to a water-soluble non-immunogenic polymer by a hydrolyzable carbamate bond. As described therein, the biologically active agent can be readily released by the hydrolysis of the carbamate bond *in vivo* without the need for adding enzymes or catalytic materials.

[0009] Another approach for reversible PEGylation is described in Peleg-Schulman (2004) *J. Med. Chem.* 47:4897-4904, WO 2004/089280 and U.S. Patent Application Publication No. 2006/0171920. Yet another releasable approach is described in U.S. Patent Application Publication No. 2006/0293499.

[0010] In the area of neutropenia, G-CSF can sometimes be administered to a patient to address or otherwise ameliorate this disorder. Filgrastim and pegfilgrastim (available as NEUOPOGEN[®] and NEULASTA[®], respectively, each from Amgen Inc., Thousand Oaks, CA) are used in the treatment of patients suffering from neutropenia. With respect to pegfilgrastim, a stable bond connects G-CSF to a linear poly(ethylene glycol). Despite the commercial success of this product, additional options for providing compositions having G-CSF activity *in vivo* would provide clinicians with a better ability to customize or tailor treatment to individual patients. Thus, the present disclosure seeks to solve this and other needs in the art.

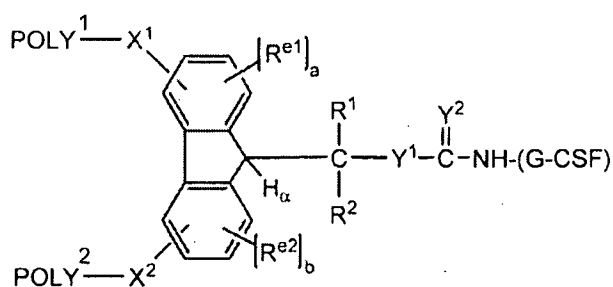
SUMMARY

[0011] In one or more first aspects, provided is a composition comprising a G-CSF moiety releasably attached to a branched water-soluble polymer at from one to three of its amino sites via a linkage selected from -O-C(O)-NH-, -O-C(S)-NH-, -S-C(O)-NH-, and -S-C(S)-NH-, wherein the NH- indicates an amino group on the G-CSF moiety to which the branched water soluble polymer is releasably attached.

[0012] In one embodiment related to the first aspect, the composition substantially comprises the G-CSF moiety releasably attached to the branched water-soluble polymer at only two of its amino sites.

[0013] In a further embodiment related to the first aspect, the composition substantially comprises the G-CSF moiety releasably attached to the branched water-soluble polymer at only a single amino site of the G-CSF moiety.

[0014] A further embodiment related to the first aspect, the composition comprises a G-CSF moiety conjugate in accordance with the following structure:



(I)

wherein:

- POLY¹ is a first water-soluble polymer;
- POLY² is a second water-soluble polymer;
- X¹ is a first spacer moiety;
- X² is a second spacer moiety;
- H_α is an ionizable hydrogen atom;
- R¹ is H, SO₃H, or an organic radical;
- R² is H, SO₃H, or an organic radical;
- (a) is either zero or one;

(b) is either zero or one;

R^{e1} , when present, is a first electron altering group;

R^{e2} , when present, is a second electron altering group; and

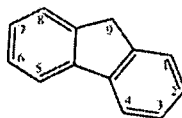
Y^1 is O or S;

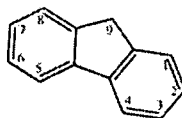
Y^2 is O or S; and

(G-CSF) is a residue of an amine-containing G-CSF moiety (where the NH-G-CSF indicates the amine-residue thereof).

[0015] Compositions comprising any one or more of the following conjugates are also contemplated.

[0016] Releasable conjugates in accordance with the foregoing structure may possess the $POLY^1-X^1$ - and $POLY^2-X^2$ - arms at any two available positions within the central fluorenyl



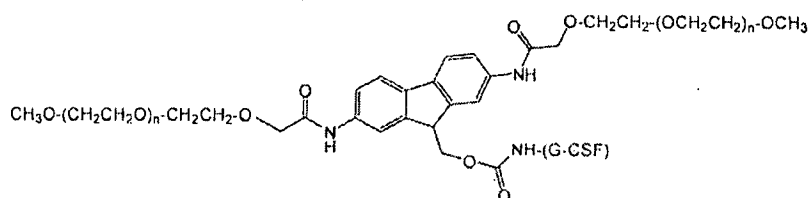
scaffold. That is to say, in reference to the scaffold , the $POLY-X$ groups may be attached at any two positions within the two phenyl rings where $POLY^1-X^1$ is at a position selected from 5, 6, 7, and 8 and $POLY^2-X^2$ is at a position selected from 1, 2, 3, and 4. Each and every combination therein is explicitly contemplated.

[0017] Representative X^1 and X^2 spacer moieties are independently selected from $-NHC(O)-(CH_2)_{1-6}-$, $-NHC(O)(CH_2)_{1-6}C(O)NH(CH_2)_2-$, $-C(O)-O-(CH_2)_{1-6}-$, $-(CH_2)_{1-6}C(O)NH(CH_2)_2-$, $-C(O)NH-(CH_2)_2-$ and $-C(O)NH-(CH_2)_2-$, wherein the spacers can be in either orientation with respect to covalent attachment to the fluorenyl core and the water-soluble polymer.

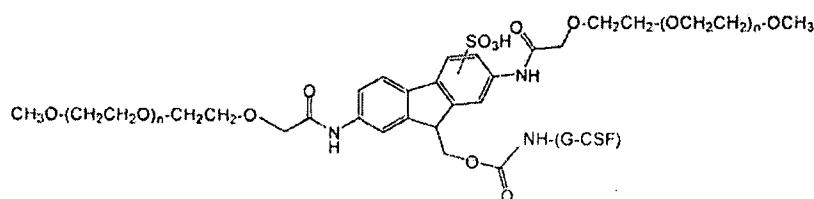
[0018] In one embodiment, the X^1 and X^2 spacer moieties are independently selected from $-NHC(O)-(CH_2)-$, $-NHC(O)-(CH_2)_2-$, $-NHC(O)-(CH_2)_3-$, $-NHC(O)-(CH_2)_4-$, $-NHC(O)-(CH_2)_5-$, $-NHC(O)-(CH_2)_6-$, $-NHC(O)(CH_2)C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_2C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_3C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_4C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_5C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_6C(O)NH(CH_2)_2-$, $-C(O)-O-(CH_2)-$, $-C(O)-O-(CH_2)_2-$, $-C(O)-O-(CH_2)_3-$, $-C(O)-O-(CH_2)_4-$, $-C(O)-O-(CH_2)_5-$, $-C(O)-O-(CH_2)_6-$, $-(CH_2)C(O)NH(CH_2)_2-$, $-(CH_2)_2C(O)NH(CH_2)_2-$, $-(CH_2)_3C(O)NH(CH_2)_2-$, $-(CH_2)_4C(O)NH(CH_2)_2-$, $-(CH_2)_5C(O)NH(CH_2)_2-$, $-(CH_2)_6C(O)NH(CH_2)_2-$, and $-C(O)NH-(CH_2)_2-$.

[0019] In one or more embodiments, the X^1 and X^2 spacer moieties are the same.

- [0020] In yet another embodiment, the X^1 and X^2 spacer moieties are different.
- [0021] In yet another embodiment, the X^1 and X^2 spacer moieties are both and $-\text{C}(\text{O})\text{NH}-(\text{CH}_2)_2-$.
- [0022] In an additional embodiment, X^1 is $-\text{C}(\text{O})\text{NH}-(\text{CH}_2)_2-$ and X^2 is $\text{NHC}(\text{O})(\text{CH}_2)_3\text{C}(\text{O})\text{NH}(\text{CH}_2)_2-$.
- [0023] In yet another embodiment, R^1 and R^2 are both H.
- [0024] In yet a further embodiment, Y^1 is O.
- [0025] In yet a further embodiment, Y^2 is O.
- [0026] In yet another embodiment, R^{e1} , when present, is an electron-withdrawing group.
- [0027] In a still further embodiment, R^{e2} , when present, is an electron-withdrawing group.
- [0028] In yet an additional embodiment, each of R^{e1} and R^{e2} , if present, either singly or collectively, is independently selected from a halogen, $-\text{C}(\text{O})\text{H}$, $-\text{C}(\text{O})\text{R}$, $-\text{C}(\text{O})\text{OR}$, $-\text{C}(\text{O})\text{OH}$, $-\text{C}(\text{O})\text{X}$, where X is a halo group, $-\text{CF}_3$, $-\text{C}\equiv\text{N}$, and $-\text{SO}_3\text{H}$, where R is an organic radical. Preferred organic radicals include alkyl radicals, substituted alkyl radicals, and more preferably, lower alkyl radicals.
- [0029] In yet a further embodiment, R^{e1} is $-\text{SO}_3\text{H}$.
- [0030] In yet another embodiment, the conjugate is selected from:



(II)



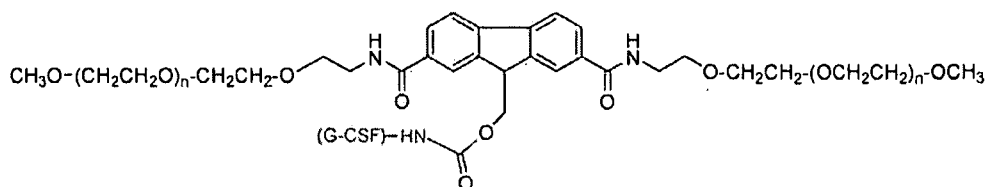
(III)



[0032] In a further embodiment, R^{e2}, when present, is an electron-donating group.

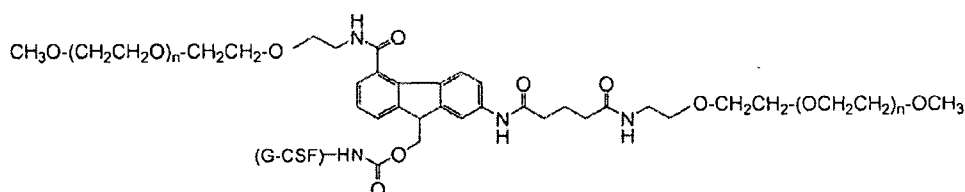
[0033] In yet an additional embodiment, each of R^{e1} and R^{e2} , if present, either singly or collectively, is independently selected from $-NH_2$, $-NRH$, $-NR_2$, $-OH$, $-OR$, and $-NHC(O)R$, where R is an organic radical, preferably an alkyl or substituted alkyl group, more preferably a lower alkyl group.

[0034] In a preferred embodiment, the conjugate possesses a structure selected from



(VII),

and



(V)

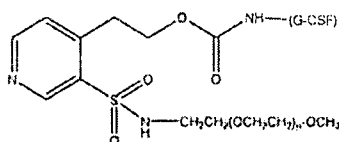
wherein, for each structure and in each instance, (n) is independently an integer from 4 to 1500, and (G-CSF) is a residue of a G-CSF moiety.

[0035] In yet another embodiment, the conjugate is a mono-G-CSF conjugate where the G-CSF moiety is covalently attached to the fluorenyl-based polymer at only a single amino site.

[0036] In yet a further embodiment, the G-CSF mono-conjugate comprises full length recombinant G-CSF covalently releasably attached to a PEG moiety as provided herein at a lysine group selected from lysine 17, lysine 24, lysine 35, lysine 41 and the N-terminus.

[0037] In yet another embodiment, the conjugate is a di-G-CSF conjugate where the G-CSF moiety is covalently attached to the fluorenyl-based polymer at two different amino sites.

[0038] In a second aspect, the releasable G-CSF conjugate possesses the structure:



wherein (n) is an integer from 4 to 1500 and (G-CSF) is a residue of a G-CSF moiety.

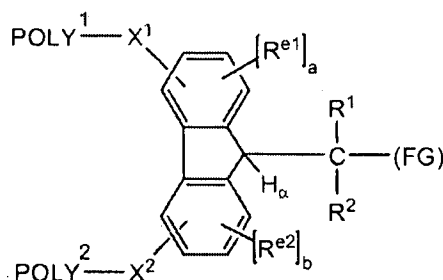
[0039] In an embodiment related to the second aspect, the releasable G-CSF conjugate is a mono-conjugate.

[0040] In yet another embodiment related to the second aspect, the releasable G-CSF conjugate is a di- or tri-conjugate.

[0041] In one or more third aspects, methods for preparing a G-CSF conjugate are provided.

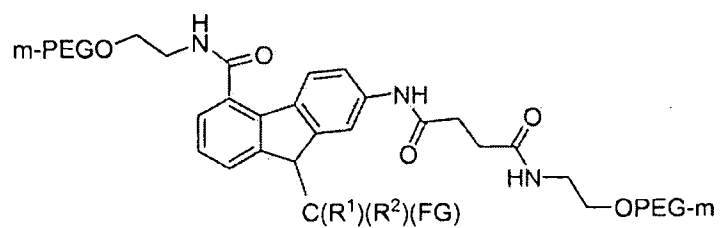
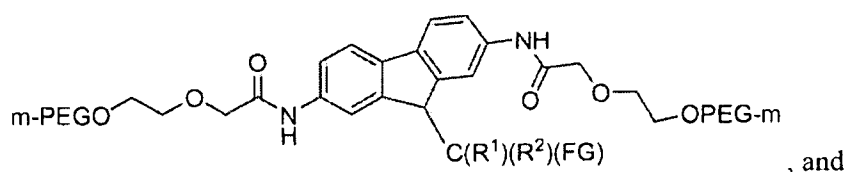
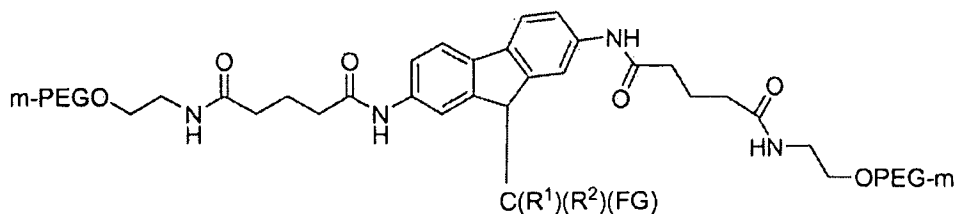
[0042] In one embodiment of the third aspect, a method for preparing a G-CSF moiety conjugate comprises: contacting an amine-reactive water-soluble polymeric reagent suitable for forming a releasable covalent bond to an amino group with a G-CSF moiety comprising at least one amino group under reaction conditions suitable for forming a releasable covalent attachment between the polymeric reagent and the G-CSF moiety.

[0043] In a particular embodiment related to the above method, the polymeric reagent has the following structure:



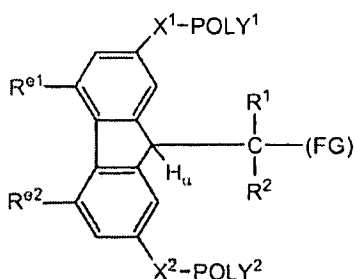
wherein POLY¹ is a first water-soluble polymer; POLY² is a second water-soluble polymer; X¹ is a first spacer moiety; X² is a second spacer moiety; H_α is an ionizable hydrogen atom; R¹ is H or an organic radical; R² is H or an organic radical; (a) is either zero or one; (b) is either zero or one; R^{e1}, when present, is a first electron altering group; R^{e2}, when present, is a second electron altering group; and (FG) is a functional group capable of reacting with an amino group of a G-CSF moiety to form a releasable linkage, such as a carbamate linkage.

[0044] In yet another preferred embodiment, the polymeric reagent has a structure selected from the group consisting of:



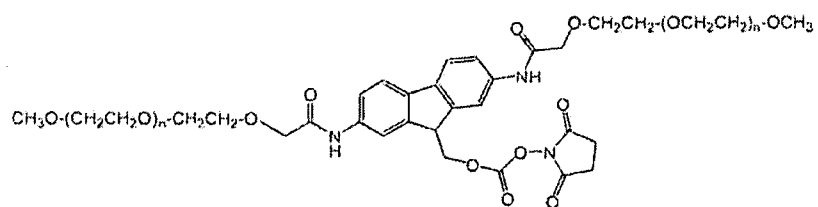
wherein, in each instance: (FG) is a functional group capable of reacting with an amino group of a G-CSF moiety to form a releasable linkage such as a carbamate linkage; R^1 is H, SO_3H , or an organic radical; and R^2 is H, SO_3H , or an organic radical.

[0045] Still, in yet one or more additional embodiments, exemplary and preferred polymeric reagents correspond to the structure:

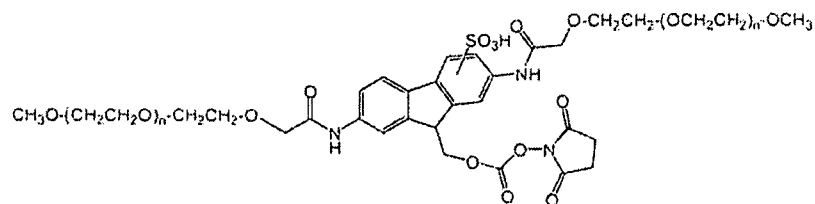


wherein each of POLY^1 , POLY^2 , X^1 , X^2 , R^1 , R^2 , H^a and (FG) is as previously defined, and R^{e1} is a first electron altering group; and R^{e2} is a second electron altering group.

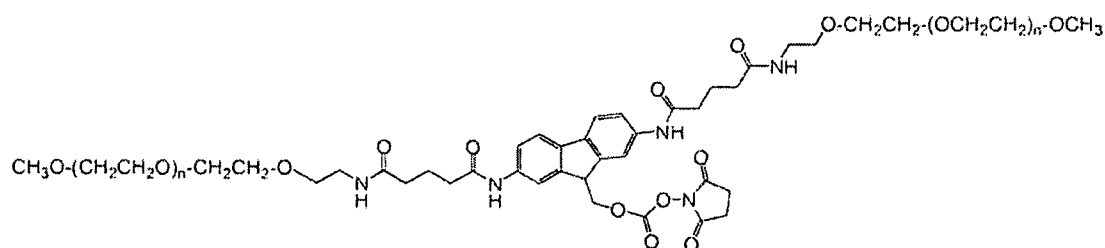
[0046] In a further embodiment, other exemplary and preferred polymeric reagents are selected from:



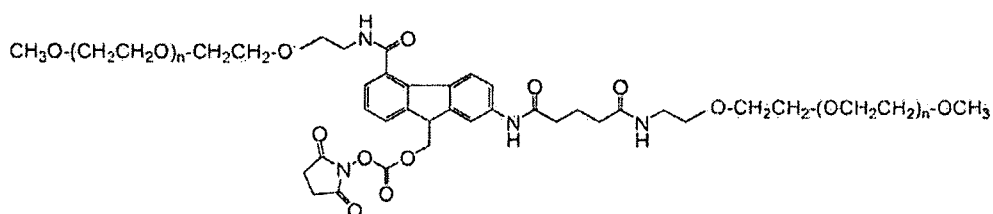
;



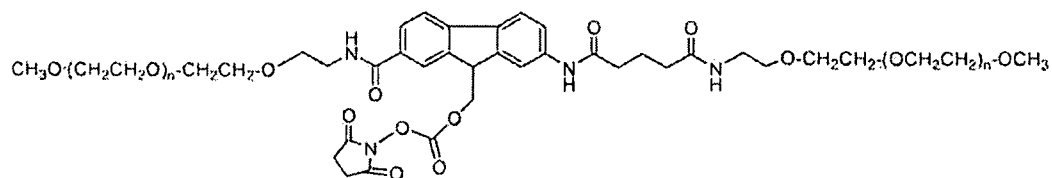
;



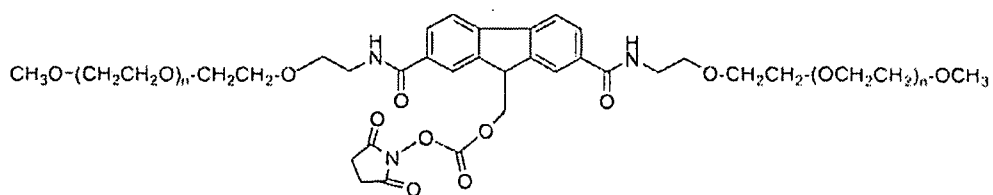
;



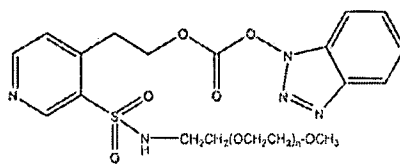
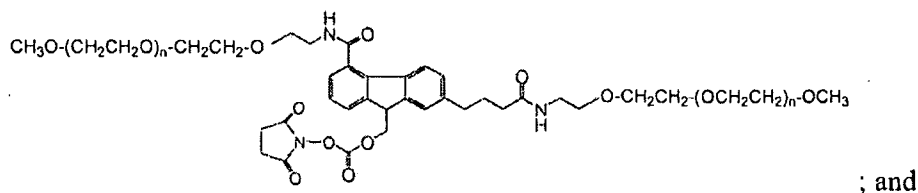
;



;



;



wherein, for each structure and in each instance, (n) is independently an integer from 4 to 1500.

[0047] In yet another embodiment of the method, the polymeric reagent is contacted with the G-CSF moiety at an equimolar amount (based upon moles of amine-reactive groups in the polymeric reagent versus moles of reactive amines in the G-CSF moiety).

[0048] In yet an alternative embodiment of the method, the polymeric reagent is contacted with the G-CSF moiety at a molar excess based upon moles of amine-reactive groups in the polymeric reagent versus moles of reactive amines in the G-CSF moiety. Thus, in this context, molar basis is determined on a reactive group basis.

[0049] In a specific embodiment, the polymeric reagent is contacted with the G-CSF moiety at a molar ratio (based upon reactive groups) of about 1:1 to 10:1.

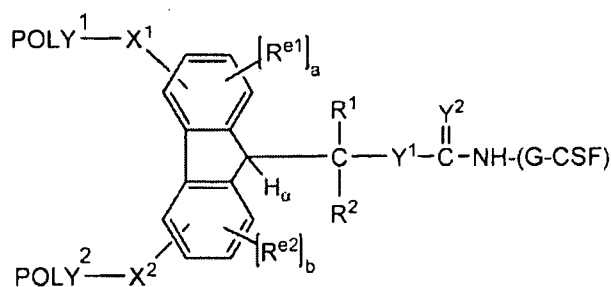
[0050] In yet another embodiment, the coupling reaction is carried out in an aqueous buffer.

[0051] In yet a further embodiment, the coupling reaction is carried out in an aqueous buffer at a pH from about 7 to about 9.

[0052] In a preferred embodiment, the reaction is carried out at room temperature (i.e., 25° C).

[0053] In one or more fourth aspects, pharmaceutical preparations comprising a G-CSF conjugate such as described herein and a pharmaceutically acceptable excipient are provided.

[0054] In an embodiment related to the fourth aspect, a composition is provided comprising a mono-G-CSF conjugate having the following general structure:



(I)

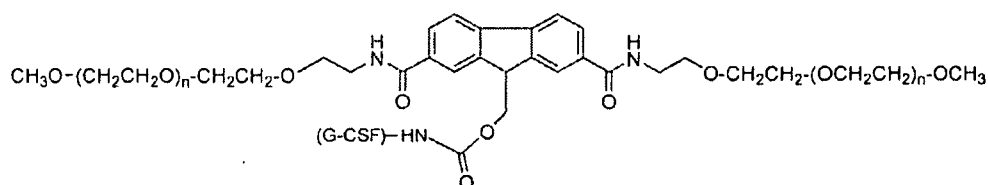
and a pharmaceutically acceptable excipient, where the variables within the structure are as described above.

[0055] In yet another embodiment related to the fourth aspect, a composition is provided comprising a mono-G-CSF conjugate having a structure selected from structures (II) - (VIII).

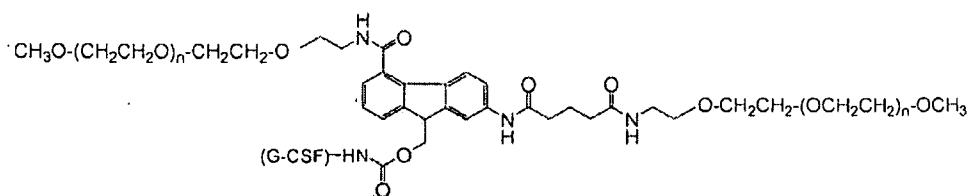
[0056] In yet another embodiment related to the fourth aspect, a composition is provided comprising a majority of a single positional isomer of a mono-G-CSF conjugate structure selected from structures (II) - (VIII), where the composition may possess additional positional isomers of the single mono-G-CSF conjugate.

[0057] In yet a further embodiment related to the fourth aspect, a composition is provided substantially comprising a single positional isomer of a mono-G-CSF conjugate selected from structures (V) and (VII), where the composition is substantially free of positional isomers of the mono-G-CSF conjugate.

[0058] In yet a further embodiment related to the fourth aspect, a composition is provided comprising a plurality of positional isomers of a single mono-G-CSF conjugate, where the mono-G-CSF conjugate is:



or



wherein, for each structure and in each instance, (n) is independently an integer from 4 to 1500 and (G-CSF) is a G-CSF moiety.

[0059] In yet a further embodiment, provided is a composition comprising a mixture of releasable mono-G-CSF conjugates, wherein each of the individual mono-G-CSF conjugates comprised in the mixture possesses a different release rate of G-CSF in-vitro under physiological conditions.

[0060] In yet a further embodiment related to the foregoing, each of the releasable mono-conjugates in the composition comprises a carbamate linkage to the G-CSF moiety.

[0061] In one or more aspects of the invention, a method for administering a G-CSF conjugate is provided.

[0062] Additional embodiments of the present conjugates, methods, compositions, and the like will be apparent from the following description, drawings, examples, and claims. As can be appreciated from the foregoing and following description, each and every feature or steps described herein, and each and every combination of two or more of such features or steps, is included within the scope of the present disclosure provided that the features/steps included in such a combination are not mutually inconsistent. In addition, any feature or combination of features or steps may be specifically excluded from any embodiment of the present invention. Additional aspects and advantages of the present invention are set forth in the following description and claims, particularly when considered in conjunction with the accompanying examples and drawings.

BRIEF DESCRIPTION OF THE FIGURES

[0063] FIG. 1 shows a chromatogram produced during cation-exchange chromatography as further described in connection with Examples 1a, 1b, 1c and 1d.

[0064] FIG. 2 shows a chromatogram produced during cation-exchange chromatography as further described in connection with Examples 2a, 2b, 2c and 2d.

[0065] **FIG. 3** shows an exemplary reverse phase HPLC chromatogram of an aliquot of a mono-conjugate composition, CG-FMOC-PEG2-40K-GCSF-M, in buffer, demonstrating the releasable nature of the mono-conjugate composition under physiological conditions as further described in Example 3.

[0066] **FIG. 4** shows an exemplary reverse phase HPLC chromatogram of an aliquot of a mono-conjugate composition, C2-FMOC-PEG2-40K-GCSF-M, in buffer, demonstrating the releasable nature of the mono-conjugate composition under physiological conditions as further described in Example 3.

[0067] **FIG. 5A** shows the release profile of an exemplary G-CSF mono-conjugate composition, CG-FMOC-PEG2-40K-GCSF-M, in PBS at pH 7.4 over time; **FIG. 5B** provides a linear plot of the release rate of CG-FMOC-PEG2-40K-GCSF-M in PBS at pH 7.4 over time, as further described in Example 3.

[0068] **FIG. 6A** shows the release profile of an exemplary G-CSF mono-conjugate composition, C2-FMOC-PEG2-40K-GCSF-M, in PBS at pH 7.4 over time; and **FIG. 6B** provides a linear plot of the release rate of C2-FMOC-PEG2-40K-GCSF-M in PBS at pH 7.4 over time as further described in Example 3.

[0069] **FIG. 7** shows a plot of the proliferation of M-NFS-60 cells at 72 hours in response to releasable conjugates as further described in detail in Example 4.

[0070] **FIG. 8** provides a plot illustrating the ability of various test articles (filgrastim, rhG-CSF, CG-FMOC-PEG2-40K-GCSF-M, C2-FMOC-PEG2-40K-GCSF-M, and a stable (i.e., non-releasable) G-CSF conjugate, PEG2-ru-40K-GCSF-M) to stimulate the proliferation of M-NFS-60 cells in vitro as described in detail in Example 4. Potency data is provided for each of the representative compositions at 72 hours.

[0071] **FIG. 9** provides a plot of EC50 results for the same comparative *in-vitro* activity study as described above for FIG. 8 and as described in detail in Example 4, and

[0072] **FIG. 10** provides the mean neutrophil counts at time points 48, 60, 72, 96 and 144 hours in neutropenic mice administered (i) Neulasta® (pegfilgrastim), (ii) CG-FMOC-PEG2-40K-GCSF-A, (iii) C2-FMOC-PEG2-40K-GCSF-A and (iv) vehicle, each at a dose of 1000 µg/kg as described in detail in Example 5.

DETAILED DESCRIPTION OF THE INVENTION

[0073] Before describing the present invention in detail, it is to be understood that this invention is not limited to particular polymers, synthetic techniques, reaction conditions, active agents, conjugates, and the like, as such may vary.

[0074] It must be noted that, as used in this specification and the claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a "polymer" includes a single polymer as well as two or more of the same or different polymers, reference to a "conjugate" refers to a single conjugate as well as two or more of the same or different conjugates, reference to an "excipient" includes a single excipient as well as two or more of the same or different excipients, and the like.

[0075] In describing and claiming the present invention, the following terminology will be used in accordance with the definitions described below.

Definitions

[0076] The term "G-CSF moiety," as used herein, refers to those peptides, polypeptides and proteins having G-CSF activity, including (for example) G-CSF. Prior to conjugation, the G-CSF moiety has at least one electrophilic group or nucleophilic group suitable for reaction with a water soluble polymer, preferably such group is an amino group. In addition, the term "G-CSF moiety" encompasses both the G-CSF moiety prior to conjugation as well as the G-CSF moiety residue following conjugation. As will be explained in further detail below, one of ordinary skill in the art can determine whether any given moiety has G-CSF activity.

[0077] As used herein, the terms "peptide," "polypeptide," and "protein," refer to polymers comprised of amino acid monomers linked by amide bonds. For use herein, each of "peptide," "polypeptide" and "protein" will be referred to as "peptide." Peptides may include the standard 20 α -amino acids that are used in protein synthesis by cells (*i.e.* natural amino acids), as well as non-natural amino acids (non-natural amino acids may be found in nature, but not used in protein synthesis by cells, *e.g.*, ornithine, citrulline, and sarcosine, or may be chemically synthesized), amino acid analogs, and peptidomimetics. The amino acids may be D- or L-optical isomers. Peptides may be formed by a condensation or coupling reaction between the α -carbon carboxyl group of one amino acid and the amino group of another amino acid. The terminal amino acid at one end of the chain (amino terminal) therefore has a free amino group, while the terminal amino acid at the other end of the chain (carboxy terminal) has a free carboxyl group.

Alternatively, the peptides may be non-linear, branched peptides or cyclic peptides. Moreover, the peptides may optionally be modified or protected with a variety of functional groups or protecting groups, including on the amino and/or carboxy terminus.

[0078] Amino acid residues in peptides are abbreviated as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G.

[0079] The terms "therapeutic peptide fragment" or "fragments of therapeutic peptides" refer to a peptide that comprises a truncation at the amino-terminus and/or a truncation at the carboxyl-terminus of a therapeutic peptide as defined herein. The terms "therapeutic peptide fragment" or "fragments of therapeutic peptides" also encompasses amino-terminal and/or carboxyl-terminal truncations of therapeutic peptide variants and therapeutic peptide derivatives. Therapeutic peptide fragments may be produced by synthetic techniques known in the art or may arise from *in vivo* protease activity on longer peptide sequences. It will be understood that therapeutic peptide fragments retain some or all of the therapeutic activities of the therapeutic peptides.

[0080] As used herein, the terms "therapeutic peptide variants" or "variants of therapeutic peptides" refer to therapeutic peptides having one or more amino acid substitutions, including conservative substitutions and non-conservative substitutions, amino acid deletions (either internal deletions and/or C- and/or N- terminal truncations), amino acid additions (either internal additions and/or C- and/or N- terminal additions, *e.g.*, fusion peptides), or any combination thereof. Variants may be naturally occurring (*e.g.* homologs or orthologs), or non-natural in origin. The term "therapeutic peptide variants" may also be used to refer to therapeutic peptides incorporating one or more non-natural amino acids, amino acid analogs, and peptidomimetics. It will be understood that, in accordance with the invention, therapeutic peptide fragments retain some or all of the therapeutic activities of the therapeutic peptides.

[0081] The terms "therapeutic peptide derivatives" or "derivatives of therapeutic peptides" as used herein refer to therapeutic peptides, therapeutic peptide fragments, and therapeutic peptide variants that have been chemically altered other than through covalent

attachment of a water-soluble polymer. It will be understood that, in accordance with the invention, therapeutic peptide derivatives retain some or all of the therapeutic activities of the therapeutic peptides.

[0082] As used herein, the terms "amino terminus protecting group" or "N-terminal protecting group," "carboxy terminus protecting group" or "C-terminal protecting group;" or "side chain protecting group" refer to any chemical moiety capable of addition to and optionally removal from a functional group on a peptide (*e.g.*, the *N*-terminus, the *C*-terminus, or a functional group associated with the side chain of an amino acid located within the peptide) to allow for chemical manipulation of the peptide.

[0083] "PEG," "polyethylene glycol" and "poly(ethylene glycol)" as used herein, are meant to encompass any water-soluble poly(ethylene oxide). Typically, PEGs for use in accordance with the invention comprise the following structure $\text{"-O(CH}_2\text{CH}_2\text{O)}_m\text{"}$ where (*m*) is 2 to 4000. As used herein, PEG also includes $\text{"-CH}_2\text{CH}_2\text{-O(CH}_2\text{CH}_2\text{O)}_m\text{-CH}_2\text{CH}_2\text{"}$ and $\text{"-(CH}_2\text{CH}_2\text{O)}_m\text{"}$, depending upon whether or not the terminal oxygens have been displaced. When the PEG further comprises a spacer moiety (to be described in greater detail below), the atoms comprising the spacer moiety, when covalently attached to a water-soluble polymer segment, do not result in the formation of an oxygen-oxygen bond (*i.e.*, an $\text{"-O-O-"$ or peroxide linkage). Throughout the specification and claims, it should be remembered that the term "PEG" includes structures having various terminal or "end capping" groups and so forth. The term "PEG" also means a polymer that contains a majority, that is to say, greater than 50%, of $\text{-CH}_2\text{CH}_2\text{O-}$ monomeric subunits. With respect to specific forms, the PEG can take any number of a variety of molecular weights, as well as structures or geometries such as "branched," "linear," "forked," "multifunctional," and the like, to be described in greater detail below.

[0084] The terms "end-capped" or "terminally capped" are interchangeably used herein to refer to a terminal or endpoint of a polymer having an end-capping moiety. Typically, although not necessarily, the end-capping moiety comprises a hydroxy or C_{1-20} alkoxy group. Thus, examples of end-capping moieties include alkoxy (*e.g.*, methoxy, ethoxy and benzyloxy), as well as aryl, heteroaryl, cyclo, heterocyclo, and the like. In addition, saturated, unsaturated, substituted and unsubstituted forms of each of the foregoing are envisioned. Moreover, the end-capping group can also be a silane. The end-capping group can also advantageously comprise a detectable label. When the polymer has an end-capping group comprising a detectable label, the amount or location of the polymer and/or the moiety (*e.g.*, active agent) of

interest to which the polymer is coupled can be determined by using a suitable detector. Such labels include, without limitation, fluorescers, chemilumescers, moieties used in enzyme labeling, colorimetric (e.g., dyes), metal ions, radioactive moieties, and the like. Suitable detectors include photometers, films, spectrometers, and the like.

[0085] "Non-naturally occurring" with respect to a polymer or water-soluble polymer means a polymer that in its entirety is not found in nature. A non-naturally occurring polymer or water-soluble polymer may, however, contain one or more subunits or portions of a subunit that are naturally occurring, so long as the overall polymer structure is not found in nature.

[0086] The term "water-soluble polymer" is any polymer that is soluble in water at room temperature. Typically, a water-soluble polymer will transmit at least about 75%, more preferably at least about 95% of light, transmitted by the same solution after filtering. On a weight basis, a water-soluble polymer will preferably be at least about 35% (by weight) soluble in water, more preferably at least about 50% (by weight) soluble in water, still more preferably about 70% (by weight) soluble in water, and still more preferably about 85% (by weight) soluble in water. It is still more preferred, however, that the water-soluble polymer is about 95% (by weight) soluble in water and most preferred that the water-soluble polymer is completely soluble in water.

[0087] Molecular weight in the context of a water-soluble polymer of the invention, such as PEG, can be expressed as either a number average molecular weight or a weight average molecular weight. Unless otherwise indicated, all references to molecular weight herein refer to the weight average molecular weight. Both molecular weight determinations, number average and weight average, can be measured using gel permeation chromatography or other liquid chromatography techniques. Other methods for measuring molecular weight values can also be used, such as the use of end-group analysis or the measurement of colligative properties (e.g., freezing-point depression, boiling-point elevation, or osmotic pressure) to determine number average molecular weight or the use of light scattering techniques, ultracentrifugation or viscometry to determine weight average molecular weight. The polymers of the invention are typically polydisperse (i.e., number average molecular weight and weight average molecular weight of the polymers are not equal), possessing low polydispersity values of preferably less than about 1.2, more preferably less than about 1.15, still more preferably less than about 1.10, yet still more preferably less than about 1.05, and most preferably less than about 1.03.

[0088] As used herein, the term "carboxylic acid" is a moiety having a $\text{-}\overset{\text{O}}{\parallel}\text{C}\text{-OH}$ functional group [also represented as a "-COOH" or -C(O)OH], as well as moieties that are derivatives of a carboxylic acid, such derivatives including, for example, protected carboxylic acids. Thus, unless the context clearly dictates otherwise, the term carboxylic acid includes not only the acid form, but corresponding esters and protected forms as well. With regard to protecting groups suited for a carboxylic acid and any other functional group described herein, reference is made to Greene *et al.*, "PROTECTIVE GROUPS IN ORGANIC SYNTHESIS" 3rd Edition, John Wiley and Sons, Inc., New York, 1999.

[0089] The terms "reactive" and "activated" when used in conjunction with a particular functional group, refer to a reactive functional group that reacts readily with an electrophile or a nucleophile on another molecule. This is in contrast to those groups that require strong catalysts or highly impractical reaction conditions in order to react (i.e., a "nonreactive" or "inert" group).

[0090] The terms "protected," "protecting group," and "protective group" refer to the presence of a moiety (i.e., the protecting group) that prevents or blocks reaction of a particular chemically reactive functional group in a molecule under certain reaction conditions. The protecting group will vary depending upon the type of chemically reactive functional group being protected as well as the reaction conditions to be employed and the presence of additional reactive or protecting groups in the molecule, if any. Protecting groups known in the art can be found in Greene *et al.*, *supra*.

[0091] As used herein, the term "functional group" or any synonym thereof is meant to encompass protected forms thereof.

[0092] The terms "spacer" or "spacer moiety" are used herein to refer to an atom or a collection of atoms optionally appearing between one moiety and another. The spacer moieties may be hydrolytically stable or may include one or more physiologically hydrolyzable or enzymatically releasable linkages.

[0093] An "organic radical" as used herein includes, for example, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl and substituted aryl. An organic radical contains at least one carbon atom.

[0094] "Alkyl" refers to a hydrocarbon chain, typically ranging from about 1 to 20 atoms in length. Such hydrocarbon chains are preferably but not necessarily saturated and may be

branched or straight chain, although typically straight chain is preferred. Exemplary alkyl groups include methyl, ethyl, propyl, butyl, pentyl, 1-methylbutyl, 1-ethylpropyl, 3-methylpentyl, and the like. As used herein, "alkyl" includes cycloalkyl when three or more carbon atoms are referenced and lower alkyl.

[0095] "Lower alkyl" refers to an alkyl group containing from 1 to 6 carbon atoms, and may be straight chain or branched, as exemplified by methyl, ethyl, *n*-butyl, *iso*-butyl, and *tert*-butyl.

[0096] "Cycloalkyl" refers to a saturated or unsaturated cyclic hydrocarbon chain, including bridged, fused, or spiro cyclic compounds, preferably made up of 3 to about 12 carbon atoms, more preferably 3 to about 8 carbon atoms.

[0097] "Non-interfering substituents" are those groups that, when present in a molecule, are typically non-reactive with other functional groups contained within the molecule.

[0098] The term "substituted" as in, for example, "substituted alkyl," refers to a moiety (e.g., an alkyl group) substituted with one or more non-interfering substituents, such as, but not limited to: C₃-C₈ cycloalkyl, e.g., cyclopropyl, cyclobutyl, and the like; halo, e.g., fluoro, chloro, bromo, and iodo; cyano; alkoxy, lower phenyl; substituted phenyl; and the like, for one or more hydrogen atoms. "Substituted aryl" is aryl having one or more non-interfering groups as a substituent. For substitutions on a phenyl ring, the substituents may be in any orientation (i.e., ortho, meta, or para). "Substituted ammonium" is ammonium having one or more non-interfering groups (e.g., an organic radical) as a substituent.

[0099] "Alkoxy" refers to an -O-R group, wherein R is alkyl or substituted alkyl, preferably C₁-C₂₀ alkyl (e.g., methoxy, ethoxy, propyloxy, benzyl, etc.), more preferably C₁-C₇ alkyl.

[0100] As used herein, "alkenyl" refers to a branched or unbranched hydrocarbon group of 2 to 15 atoms in length, containing at least one double bond. Exemplary alkenyl include (without limitation) ethenyl, *n*-propenyl, isopropenyl, *n*-butenyl, *iso*-butenyl, octenyl, decenyl, tetradecenyl, and the like.

[0101] The term "alkynyl" as used herein refers to a branched or unbranched hydrocarbon group of 2 to 15 atoms in length, containing at least one triple bond. Exemplary alkynyl include (without limitation) ethynyl, *n*-butynyl, *iso*-pentynyl, octynyl, decynyl, and so forth.

[0102] "Aryl" means one or more aromatic rings, each of 5 or 6 core carbon atoms. Aryl includes multiple aryl rings that may be fused, as in naphthyl, or unfused, as in biphenyl. Aryl rings may also be fused or unfused with one or more cyclic hydrocarbon, heteroaryl, or heterocyclic rings. As used herein, "aryl" includes heteroaryl. An aromatic-containing moiety (e.g., Ar¹, Ar², and so forth), means a structure containing aryl.

[0103] "Heteroaryl" is an aryl group containing from one to four heteroatoms, preferably N, O, or S, or a combination thereof. Heteroaryl rings may also be fused with one or more cyclic hydrocarbon, heterocyclic, aryl, or heteroaryl rings.

[0104] "Heterocycle" or "heterocyclic" means one or more rings of 5-12 atoms, preferably 5-7 atoms, with or without unsaturation or aromatic character and having at least one ring atom which is not a carbon. Preferred heteroatoms include sulfur, oxygen, and nitrogen.

[0105] "Substituted heteroaryl" is heteroaryl having one or more non-interfering groups as substituents.

[0106] "Substituted heterocycle" is a heterocycle having one or more side chains formed from non-interfering substituents.

[0107] "Electrophile" refers to an ion or atom or collection of atoms, which may be ionic, having an electrophilic center, i.e., a center that is electron seeking, capable of reacting with a nucleophile.

[0108] "Nucleophile" refers to an ion or atom or collection of atoms that may be ionic having a nucleophilic center, i.e., a center that is seeking an electrophilic center or with an electrophile.

[0109] A "physiologically cleavable" as well as a "hydrolyzable" bond is a relatively weak bond that reacts with water (i.e., is hydrolyzed) under physiological conditions. The tendency of a bond to hydrolyze in water will depend not only on the general type of linkage connecting two central atoms but also on the substituents attached to these central atoms. Exemplary hydrolyzable bonds include, but are not limited to, carboxylate ester, phosphate ester, anhydride, acetal, ketal, acyloxyalkyl ether, imine, and ortho esters.

[0110] A "releasable linkage" includes, but is not limited to, a physiologically cleavable bond, a hydrolyzable bond, and an enzymatically degradable linkage. Thus, a "releasable linkage" is a linkage that may undergo either hydrolysis or cleavage by some other mechanism

(e.g., enzyme-catalyzed, acid-catalyzed, base-catalyzed, and so forth) under physiological conditions. For example, a "releaseable linkage" can involve an elimination reaction that has a base abstraction of a proton, (e.g., an ionizable hydrogen atom, H_a), as the driving force. For purposes herein, a "releaseable linkage" is synonymous with a "degradable linkage."

[0111] An "enzymatically releasable linkage" means a linkage that is subject to degradation by one or more enzymes.

[0112] A "hydrolytically stable" linkage or bond refers to a chemical bond, typically a covalent bond, which is substantially stable in water, that is to say, does not undergo hydrolysis under physiological conditions to any appreciable extent over an extended period of time. Examples of hydrolytically stable linkages include but are not limited to the following: carbon-carbon bonds (e.g., in aliphatic chains), ethers, amides, and the like. Generally, a hydrolytically stable linkage is one that exhibits a rate of hydrolysis of less than about 1-2% per day under physiological conditions. Hydrolysis rates of representative chemical bonds can be found in most standard chemistry textbooks. It must be pointed out that some linkages can be hydrolytically stable or hydrolyzable, depending upon (for example) adjacent and neighboring atoms and ambient conditions. One of ordinary skill in the art can determine whether a given linkage or bond is hydrolytically stable or hydrolyzable in a given context by, for example, placing a linkage-containing molecule of interest under conditions of interest and testing for evidence of hydrolysis (e.g., the presence and amount of two molecules resulting from the cleavage of a single molecule). Other approaches known to those of ordinary skill in the art for determining whether a given linkage or bond is hydrolytically stable or hydrolyzable can also be used.

[0113] The terms "active agent," "biologically active agent" and "pharmacologically active agent" are used interchangeably herein and are defined to include any agent, drug, compound, composition of matter or mixture that provides some pharmacologic, often beneficial, effect that can be demonstrated *in vivo* or *in vitro*. This includes food supplements, nutrients, nutraceuticals, drugs, proteins, vaccines, antibodies, vitamins, and other beneficial agents. As used herein, these terms further include any physiologically or pharmacologically active substance that produces a localized or systemic effect in a patient.

[0114] "Pharmaceutically acceptable excipient" or "pharmaceutically acceptable carrier" refers to an excipient that can be included in the compositions of the invention and that causes no significant adverse toxicological effects to the patient.

[0115] "Pharmacologically effective amount," "physiologically effective amount," and "therapeutically effective amount" are used interchangeably herein to mean the amount of a polymer-active agent conjugate -- typically present in a pharmaceutical preparation -- that is needed to provide a desired level of active agent and/or conjugate in the bloodstream or in a target tissue. The exact amount will depend upon numerous factors, e.g., the particular active agent, e.g., G-CSF, the components and physical characteristics of the pharmaceutical preparation, intended patient population, patient considerations, and the like, and can readily be determined by one of ordinary skill in the art, based upon the information provided herein and available in the relevant literature.

[0116] "Multifunctional" in the context of a polymer means a polymer having 3 or more functional groups contained therein, where the functional groups may be the same or different. Multifunctional polymers will typically contain from about 3-100 functional groups, or from 3-50 functional groups, or from 3-25 functional groups, or from 3-15 functional groups, or from 3 to 10 functional groups, or will contain 3, 4, 5, 6, 7, 8, 9 or 10 functional groups within the polymer. A "difunctional" polymer means a polymer having two functional groups contained therein, either the same (i.e., homodifunctional) or different (i.e., heterodifunctional).

[0117] "Branched," in reference to the geometry or overall structure of a polymer, refers to polymer having 2 or more polymer "arms." A branched polymer may possess 2 polymer arms, 3 polymer arms, 4 polymer arms, 6 polymer arms, 8 polymer arms or more. One particular type of highly branched polymer is a dendritic polymer or dendrimer, which, for the purposes of the invention, is considered to possess a structure distinct from that of a branched polymer.

[0118] A "dendrimer" or dendritic polymer is a globular, size monodisperse polymer in which all bonds emerge radially from a central focal point or core with a regular branching pattern and with repeat units that each contribute a branch point. Dendrimers exhibit certain dendritic state properties such as core encapsulation, making them unique from other types of polymers.

[0119] A basic or acidic reactant described herein includes neutral, charged, and any corresponding salt forms thereof.

[0120] The term "patient," refers to a living organism suffering from or prone to a condition that can be prevented or treated by administration of a conjugate as provided herein, and includes both humans and animals.

[0121] As used herein, "drug release rate" means a rate (stated as a half-life) in which half of the total amount of polymer-active agent conjugate(s) in a system will cleave under a given set of conditions to release the active agent and a polymeric residue.

[0122] "Optional" and "optionally" mean that the subsequently described circumstance may or may not occur, so that the description includes instances where the circumstance occurs and instances where it does not.

[0123] "Substantially" or "essentially" means nearly totally or completely, for instance, 95% or greater of some given quantity.

[0124] A "minor" amount refers to 5% or less of some given quantity. A minor amount may, e.g., range from less than 5% to 0.1% of some given quantity.

[0125] A "majority" of a particular item refers to greater than 50% of such item out of the population under consideration. For example, a composition that contains a majority of a single positional isomer of a G-CSF mono-conjugate is one that contains greater than 50% of such mono-conjugate out of a population that includes the other possible positional isomers of the mono-conjugate. Thus, a majority is necessarily greater than 50% of a given population, but may, in actuality be present in an amount greater than 60%, greater than 70%, greater than 80%, or even greater than 90%.

[0126] As used herein, the "halo" designator (e.g., fluoro, chloro, iodo, bromo, and so forth) is generally used when the halogen is attached to a molecule, while the suffix "ide" (e.g., fluoride, chloride, iodide, bromide, and so forth) is used when the halogen exists in its independent ionic form (e.g., such as when a leaving group leaves a molecule).

[0127] In the context of the present discussion, it should be recognized that the definition of a variable provided with respect to one structure or formula is applicable to the same variable repeated in a different structure, unless the context dictates otherwise.

[0128] As previously stated, the present invention comprises (among other things) G-CSF conjugates comprising a releasable linkage.

Water Soluble Polymer

[0129] Before describing exemplary conjugates of the invention, embodiments of suitable water-soluble polymers and functional groups capable of reacting with an amino group of a G-CSF moiety to form a releasable linkage, such as a carbamate linkage, will be discussed.

[0130] With respect to a given water-soluble polymer, each water-soluble polymer (e.g., POLY, POLY¹ and POLY²) can comprise any polymer so long as the polymer is water-soluble and non-peptidic. Although preferably a poly(ethylene glycol), a water-soluble polymer for use in forming a G-CSF conjugate as described herein can be, for example, other water-soluble polymers such as other poly(alkylene glycols) (also referred to as "poly(alkyleneoxides)"), such as poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α -hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), such as described in U.S. Patent No. 5,629,384. The water soluble polymer can be a homopolymer, copolymer, terpolymer, nonrandom block polymer, and random block polymer of any of the foregoing. In addition, a water-soluble polymer can be linear, but can also be in other forms (e.g., branched, forked, and the like) as will be described in further detail below. In the context of being present within an overall structure, a water-soluble polymer has from 1 to about 300 termini, and preferably from 1-25 termini and even more preferably from 1-10 termini.

[0131] In instances where the polymeric reagent comprises two or more water-soluble polymer segments, each water-soluble polymer in the overall structure can be the same or different. It is preferred, however, that all water-soluble polymers in the overall structure of the polymeric reagent (and resulting conjugate) are of the same type. For example, it is preferred that all water-soluble polymers within a given structure are poly(ethylene glycol) polymers.

[0132] Although the weight-average molecular weight of any individual water-soluble polymer can vary, the weight average molecular weight of any given water-soluble polymer will typically be in a range of about 100 Daltons to about 150,000 Daltons. Exemplary ranges, however, include weight-average molecular weights in the following ranges: in the range of from about 880 Daltons to about 5,000 Daltons; in the range of greater than 5,000 Daltons to about 100,000 Daltons; in the range of from about 6,000 Daltons to about 90,000 Daltons; in the range of from about 10,000 Daltons to about 85,000 Daltons; in the range of greater than 10,000

Daltons to about 85,000 Daltons; in the range of from about 20,000 Daltons to about 85,000 Daltons; in the range of from about 53,000 Daltons to about 85,000 Daltons; in the range of from about 25,000 Daltons to about 120,000 Daltons; in the range of from about 29,000 Daltons to about 120,000 Daltons; in the range of from about 35,000 Daltons to about 120,000 Daltons; in the range of about 880 Daltons to about 60,000 Daltons; in the range of about 440 Daltons to about 40,000 Daltons; in the range of about 440 Daltons to about 30,000 Daltons; and in the range of from about 40,000 Daltons to about 120,000 Daltons. For any given water-soluble polymer, PEGs having a molecular weight in one or more of these ranges are preferred. For the branched fluorenyl-based structures described herein, the foregoing ranges are for the overall polymeric reagent; molecular weights for each of POLY¹ and POLY² are considered to be within preferred molecular weight ranges that correspond to half of each of the above. For example, a fluorenyl-based releasable polymeric reagent as described herein having a molecular weight ranging from about 880 Daltons to about 5,000 Daltons will possess a POLY¹ and a POLY² in the overall polymeric reagent each having a molecular weight ranging from about 440 Daltons to about 2,500 Daltons, and so forth.

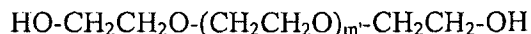
[0133] Exemplary weight-average molecular weights for the water-soluble polymeric reagent include about 100 Daltons, about 200 Daltons, about 300 Daltons, about 400 Daltons, about 440 Daltons, about 500 Daltons, about 600 Daltons, about 700 Daltons, about 750 Daltons, about 800 Daltons, about 900 Daltons, about 1,000 Daltons, about 1,500 Daltons, about 2,000 Daltons, about 2,200 Daltons, about 2,500 Daltons, about 3,000 Daltons, about 4,000 Daltons, about 4,400 Daltons, about 4,500 Daltons, about 5,000 Daltons, about 5,500 Daltons, about 6,000 Daltons, about 7,000 Daltons, about 7,500 Daltons, about 8,000 Daltons, about 9,000 Daltons, about 10,000 Daltons, about 11,000 Daltons, about 12,000 Daltons, about 13,000 Daltons, about 14,000 Daltons, about 15,000 Daltons, about 16,000 Daltons, about 17,000 Daltons, about 18,000 Daltons, about 19,000 Daltons, about 20,000 Daltons, about 22,500 Daltons, about 25,000 Daltons, about 30,000 Daltons, about 35,000 Daltons, about 40,000 Daltons, about 45,000 Daltons, about 50,000 Daltons, about 55,000 Daltons, about 60,000 Daltons, about 65,000 Daltons, about 70,000 Daltons, and about 75,000 Daltons. Branched versions of the water-soluble polymer (e.g., a branched 40,000 Dalton water-soluble polymer comprised of two 20,000 Dalton polymers) having a total weight average molecular weight of any of the foregoing can also be used.

[0134] The polymeric reagent used to prepare the G-CSF conjugate will comprise at least one water-soluble polymer having a total size in the range suited for the desired rate of release of the conjugate formed therefrom. For example, a conjugate having a relatively long release rate can be prepared from a polymeric reagent having a size suited for (a) extended circulation prior to release of the G-CSF moiety from the conjugate, and (b) moderately rapid *in vivo* clearance of the species liberated from the conjugate upon release from the conjugate. Likewise, when the conjugate has a relatively fast release rate, then the polymeric reagent would typically have a lower molecular weight.

[0135] When a PEG is used as the water-soluble polymer(s) in the polymeric reagent, the PEG typically comprises a number of $(\text{OCH}_2\text{CH}_2)$ monomers [or $(\text{CH}_2\text{CH}_2\text{O})$ monomers, depending on how the PEG is defined]. As used throughout the description, the number of repeating units is identified by the subscript "*n*" in " $(\text{OCH}_2\text{CH}_2)_n$." Thus, the value of (*n*) typically falls within one or more of the following ranges: from 2 to about 3400, from about 4 to about 1500, from about 100 to about 2300, from about 100 to about 2270, from about 136 to about 2050, from about 225 to about 1930, from about 450 to about 1930, from about 1200 to about 1930, from about 568 to about 2727, from about 660 to about 2730, from about 795 to about 2730, from about 795 to about 2730, from about 909 to about 2730, and from about 1,200 to about 1,900. For any given polymer in which the molecular weight is known, it is possible to determine the number of repeating units (i.e., "*n*") by dividing the total weight-average molecular weight of the polymer by the molecular weight of the repeating monomer.

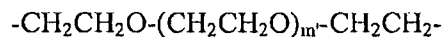
[0136] Each water-soluble polymer is typically biocompatible and non-immunogenic. With respect to biocompatibility, a substance is considered biocompatible if the beneficial effects associated with use of the substance alone or with another substance (e.g., an active agent) in connection with living tissues (e.g., administration to a patient) outweighs any deleterious effects as evaluated by a clinician, e.g., a physician. With respect to non-immunogenicity, a substance is considered non-immunogenic if use of the substance alone or with another substance in connection with living tissues does not produce an immune response (e.g., the formation of antibodies) or, if an immune response is produced, that such a response is not deemed clinically significant or important as evaluated by a clinician. It is particularly preferred that the water-soluble polymers described herein as well as conjugates of active agents and the polymers are biocompatible and non-immunogenic.

[0137] In one form useful, free or nonbound PEG is a linear polymer terminated at each end with hydroxyl groups:



wherein (m') typically ranges from zero to about 4,000, preferably from about 20 to about 1,000.

[0138] The above polymer, alpha-,omega-dihydroxypoly(ethylene glycol), can be represented in brief form as HO-PEG-OH where it is understood that the -PEG- symbol can represent the following structural unit:



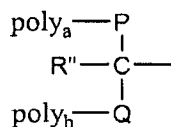
where (m') is as defined as above.

[0139] Another type of free or nonbound PEG useful in the present invention is methoxy-PEG-OH, or mPEG in brief, in which one terminus is the relatively inert methoxy group, while the other terminus is a hydroxyl group. The structure of mPEG is given below.



where (m') is as described above.

[0140] Multi-armed or branched PEG molecules, such as those described in U.S. Patent No. 5,932,462, can also be used as the PEG polymer. For example, PEG can have the structure:



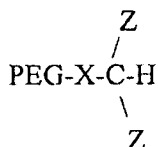
wherein:

poly_a and poly_b are PEG backbones (either the same or different), such as methoxy poly(ethylene glycol);

R'' is a nonreactive moiety, such as H, methyl or a PEG backbone; and

P and Q are nonreactive linkages. In a preferred embodiment related to the foregoing, the branched PEG polymer is methoxy poly(ethylene glycol) disubstituted lysine.

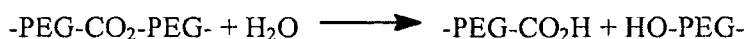
[0141] In addition, the PEG can comprise a forked PEG. An example of a free or nonbound forked PEG is represented by the following formula:



wherein: X is a spacer moiety and each Z is an activated terminal group linked to CH by a chain of atoms of defined length. The chain of atoms linking the Z functional groups to the branching carbon atom serve as a tethering group and may comprise, for example, alkyl chains, ether chains, ester chains, amide chains and combinations thereof. U.S. Patent No. 6,362,254, discloses various forked PEG structures capable of use in the present invention.

[0142] The PEG polymer may comprise a pendant PEG molecule having reactive groups, such as carboxyl, covalently attached along the length of the PEG rather than at the end of the PEG chain. The pendant reactive groups can be attached to the PEG directly or through a spacer moiety, such as an alkylene group.

[0143] In addition to the above-described forms of PEG, each water-soluble polymer in the polymeric reagent can also be prepared with one or more weak or releasable linkages in the polymer, including any of the above described polymers. For example, PEG can be prepared with ester linkages in the polymer that are subject to hydrolysis. As shown below, this hydrolysis results in cleavage of the polymer into fragments of lower molecular weight:



[0144] Other hydrolytically releasable linkages, useful as a releasable linkage within a polymer backbone, include carbonate linkages; imine linkages resulting, for example, from reaction of an amine and an aldehyde (see, e.g., Ouchi *et al.* (1997) *Polymer Preprints* 38(1):582-3); phosphate ester linkages formed, for example, by reacting an alcohol with a phosphate group; hydrazone linkages which are typically formed by reaction of a hydrazide and an aldehyde; acetal linkages that are typically formed by reaction between an aldehyde and an alcohol; ortho ester linkages that are, for example, formed by reaction between a formate and an alcohol; amide linkages formed by an amine group, e.g., at an end of a polymer such as PEG, and a carboxyl group of another PEG chain; urethane linkages formed from reaction of, e.g., a PEG with a terminal isocyanate group and a PEG alcohol; peptide linkages formed by an amine

group, e.g., at an end of a polymer such as PEG, and a carboxyl group of a peptide; and oligonucleotide linkages formed by, for example, a phosphoramidite group, e.g., at the end of a polymer, and a 5' hydroxyl group of an oligonucleotide.

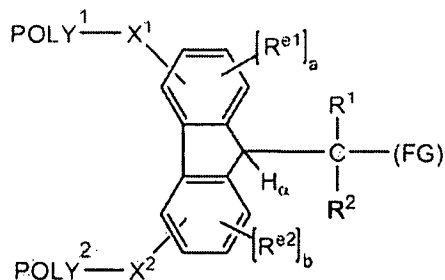
[0145] It is understood by those of ordinary skill in the art that the term poly(ethylene glycol) or PEG represents or includes all the above forms of PEG.

[0146] Those of ordinary skill in the art will recognize that the foregoing discussion concerning substantially water-soluble polymers is by no means exhaustive and is merely illustrative, and that all polymeric materials having the qualities described above are contemplated. As used herein, the term "water-soluble polymer" refers both to a molecule as well as the residue of water-soluble polymer that has been attached to another moiety. The following description of a water-soluble polymer are applicable not only to the polymeric reagent, but to the corresponding conjugates formed using the described polymeric reagents.

[0147] The functional group of the polymeric reagents used to form the conjugates described herein is a functional group capable of reacting with an amino group of a G-CSF moiety to form a releasable linkage, such as a carbamate linkage. The invention is not limited with respect to the specific functional group so long as the functional group is capable of reacting with an amino group of an active agent to form a releasable linkage, such as a carbamate linkage. Exemplary functional groups capable of reacting with an amino group of an active agent such as G-CSF include those functional groups selected from the group consisting of active carbonates such as N-succinimidyl, 1-benzotriazolyl, imidazole, carbonate halides (such as carbonate chloride and carbonate bromide), aldehydes, phenolates (such as *p*-nitrophenolate) and so forth. Also, as a special case, if the active agent is available with the active amine group converted into an isocyanate or isothiocyanate group, then the functional group of the polymeric reagent can be hydroxyl as the reaction of these components provides a releasable carbamate linkage.

[0148] Exemplary and preferred polymeric reagents will now be discussed in further detail. It must be remembered that while stereochemistry is not specifically shown in any formulae or structures (whether for a polymeric reagent, conjugate, or any other formula or structure), the provided formulae and structures contemplate all enantiomers, as well as compositions comprising mixtures of each enantiomer in equal amounts (i.e., a racemic mixture) and unequal amounts.

[0149] One exemplary and preferred polymeric reagent has the following general structure:



wherein:

POLY¹ is a first water-soluble polymer;

POLY² is a second water-soluble polymer;

X¹ is a first spacer moiety;

X² is a second spacer moiety;

H_a is an ionizable hydrogen atom;

R¹ is H or an organic radical;

R² is H or an organic radical;

(a) is either zero or one;

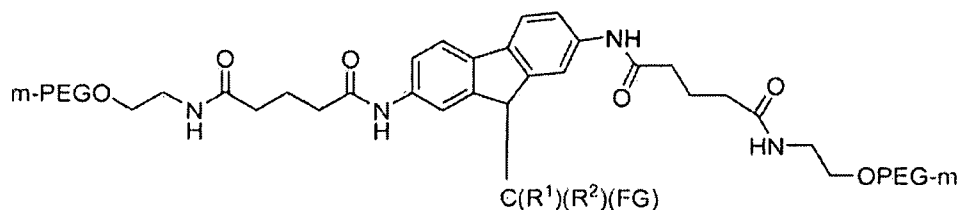
(b) is either zero or one;

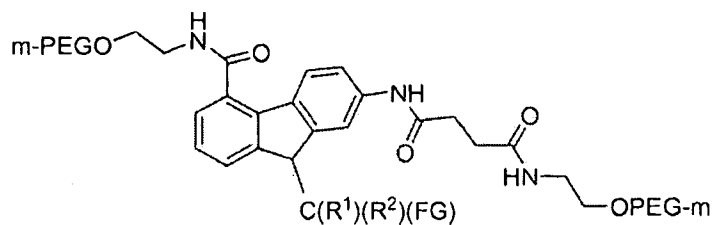
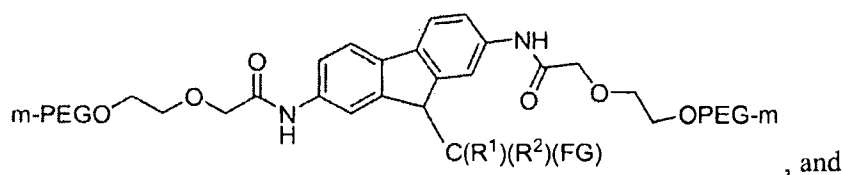
R^{e1}, when present, is a first electron altering group;

R^{e2}, when present, is a second electron altering group; and

(FG) is a functional group capable of reacting with an amino group of a G-CSF moiety to form a releasable linkage, such as a carbamate linkage.

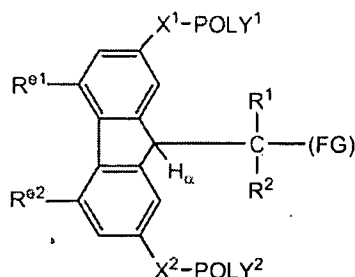
[0150] Exemplary and preferred polymeric reagents for forming a releasable G-CSF conjugate include:





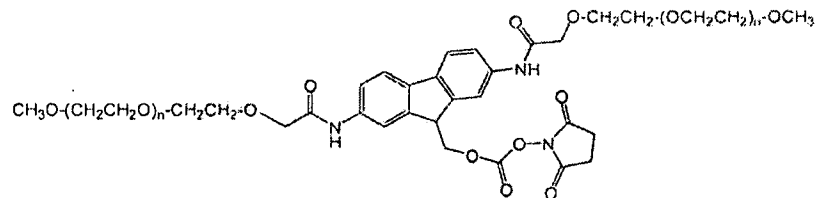
wherein, in each instance: (FG) is a functional group capable of reacting with an amino group of a G-CSF moiety to form a releasable linkage, such as a carbamate linkage; R^1 is H, SO_3H , or an organic radical; and R^2 is H, SO_3H , or an organic radical.

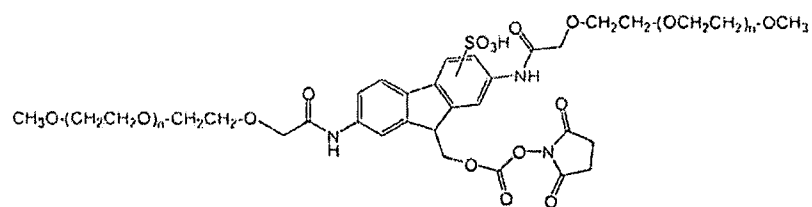
[0151] Still other exemplary and preferred polymeric reagents have the structure:



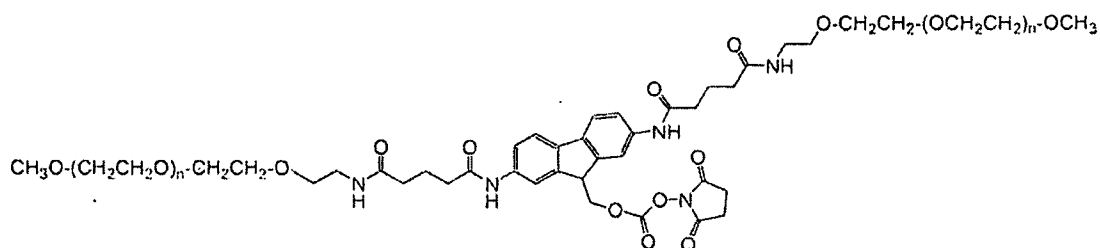
wherein each of POLY^1 , POLY^2 , X^1 , X^2 , R^1 , R^2 , H_a and (FG) is as previously defined, and $\text{R}^{\text{e}1}$ is a first electron altering group; and $\text{R}^{\text{e}2}$ is a second electron altering group.

[0152] Still other exemplary and preferred polymeric reagents fall within the following structures, although the functional group capable of reacting with an amino group of a G-CSF moiety to form a releasable linkage (e.g., the NHS-ester reactive group of the reagents illustrated below) may be substituted with any other such suitable reactive group.

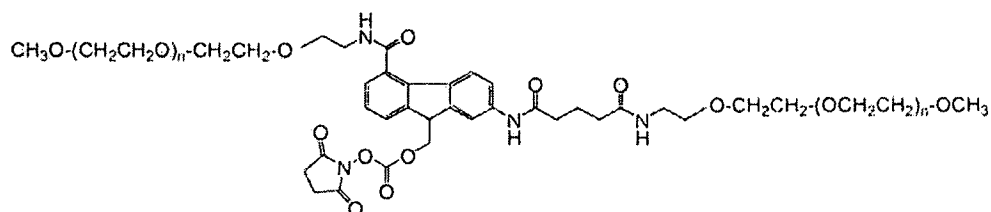




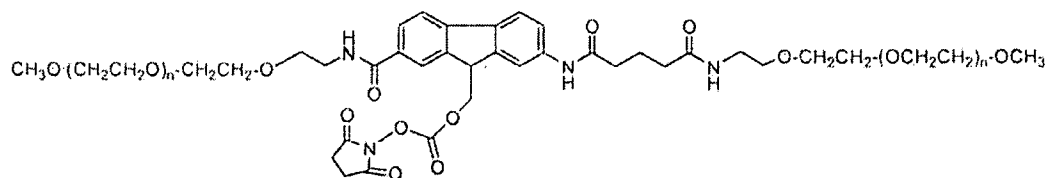
;



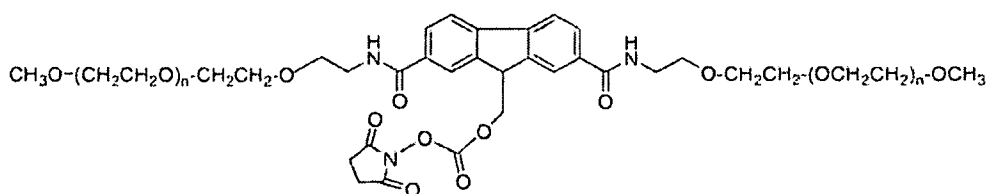
;



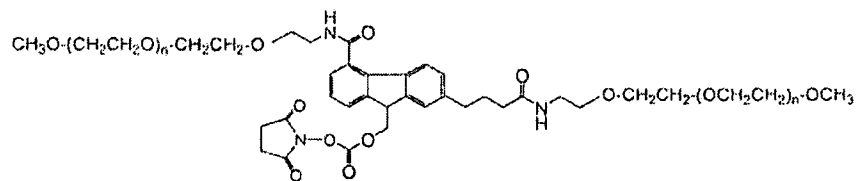
;



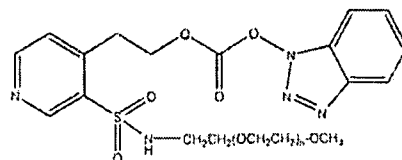
;



;



; and



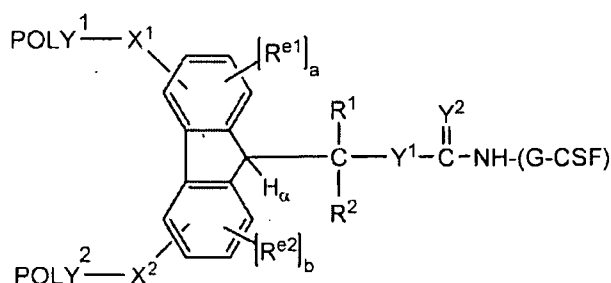
wherein, for each structure and in each instance, (n) is independently an integer from 4 to 1500.

[0153] The polymeric reagents can be prepared in any number of ways. Consequently, synthesis of the polymeric reagents is not limited to any specific technique or approach used in their preparation. Exemplary approaches for preparing the polymeric reagents are described in the literature.

[0154] The above-described polymeric reagents are particularly well-suited for conjugation to a G-CSF moiety. For example, an amino group (e.g., primary amine) on a G-CSF moiety will react with a suitable amino-reactive functional group within the polymeric reagent, to thereby form a releasable linkage such as a carbamate linkage.

Illustrative Conjugates

[0155] Exemplary releasable G-CSF conjugates include those of the following formulae:

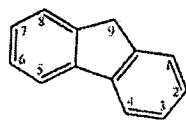


wherein:

- POLY¹ is a first water-soluble polymer;
- POLY² is a second water-soluble polymer;
- X¹ is a first spacer moiety;
- X² is a second spacer moiety;
- H_α is an ionizable hydrogen atom;
- R¹ is H or an organic radical;
- R² is H or an organic radical;

- (a) is either zero or one;
- (b) is either zero or one;
- R^{c1} , when present, is a first electron altering group;
- R^{c2} , when present, is a second electron altering group;
- Y^1 is O or S;
- Y^2 is O or S; and
- (G-CSF) is a residue of G-CSF moiety.

[0156] Releasable conjugates in accordance with the foregoing structure may possess the POLY¹ and POLY² arms at any two available positions within the central fluorenyl scaffold.



That is to say, in reference to the scaffold, the POLY groups may be at any two positions where POLY¹ is at a position selected from 5, 6, 7, and 8 and POLY² is at a position selected from 1, 2, 3, and 4. Each and every combination therein is explicitly contemplated.

[0157] Representative X^1 and X^2 spacer moieties within structure (I) are independently selected from $-NHC(O)-(CH_2)_{1-6}-$, $-NHC(O)(CH_2)_{1-6}C(O)NH(CH_2)_2-$, $-C(O)-O-(CH_2)_{1-6}-$, $-(CH_2)_{1-6}C(O)NH(CH_2)_2-$, $-C(O)NH-(CH_2)_2-$, and $-C(O)NH-(CH_2)_2-$, where the spacers can be in either orientation with respect to covalent attachment to the fluorenyl core and the water-soluble polymer.

[0158] Representative X^1 and X^2 spacer moieties include the following: $-NHC(O)-(CH_2)-$, $-NHC(O)-(CH_2)_2-$, $-NHC(O)-(CH_2)_3-$, $-NHC(O)-(CH_2)_4-$, $-NHC(O)-(CH_2)_5-$, $-NHC(O)-(CH_2)_6-$, $-NHC(O)(CH_2)C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_2C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_3C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_4C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_5C(O)NH(CH_2)_2-$, $-NHC(O)(CH_2)_6C(O)NH(CH_2)_2-$, $-C(O)-O-(CH_2)-$, $-C(O)-O-(CH_2)_2-$, $-C(O)-O-(CH_2)_3-$, $-C(O)-O-(CH_2)_4-$, $-C(O)-O-(CH_2)_5-$, $-C(O)-O-(CH_2)_6-$, $-(CH_2)C(O)NH(CH_2)_2-$, $-(CH_2)_2C(O)NH(CH_2)_2-$, $-(CH_2)_3C(O)NH(CH_2)_2-$, $-(CH_2)_4C(O)NH(CH_2)_2-$, $-(CH_2)_5C(O)NH(CH_2)_2-$, $-(CH_2)_6C(O)NH(CH_2)_2-$, and $-C(O)NH-(CH_2)_2-$.

[0159] The X^1 and X^2 spacer moieties in the conjugate may be the same, or alternatively, may be different. See, e.g., the representative structures which follow.

[0160] In one preferred G-CSF conjugate, the X^1 and X^2 spacer moieties are both $-C(O)NH-(CH_2)_2-$.

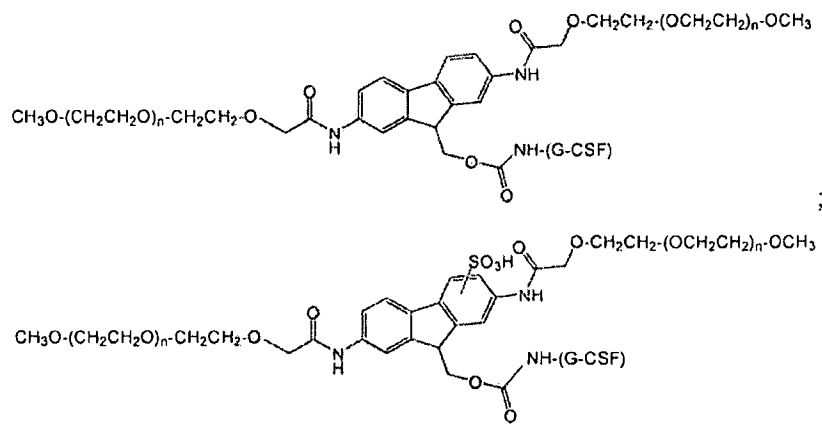
[0161] In yet an additional and preferred embodiment, X^1 is $-C(O)NH-(CH_2)_2-$ and X^2 is $NHC(O)(CH_2)_3C(O)NH(CH_2)_2-$.

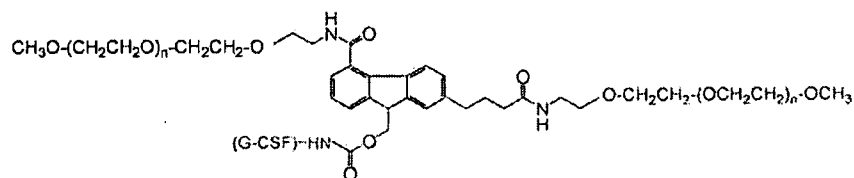
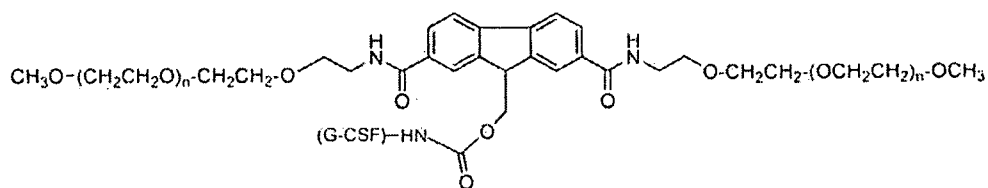
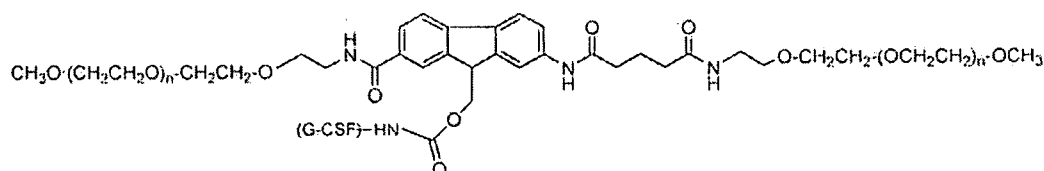
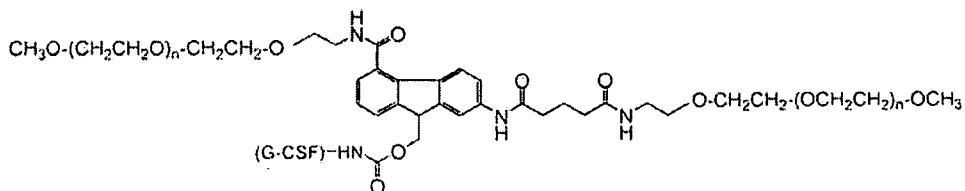
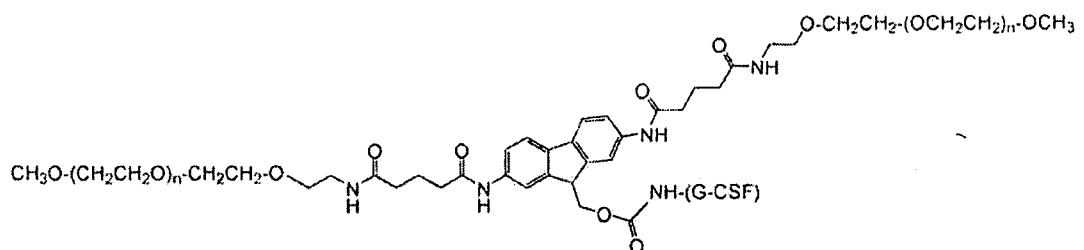
[0162] For R^1 and R^2 , each is independently H or an organic radical. Illustrative organic radicals include, for example, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl and substituted aryl. Preferred organic radicals include alkyl groups such as methyl, ethyl, propyl, butyl, pentyl, 1-methylbutyl, 1-ethylpropyl, 3-methylpentyl, and the like, where a lower alkyl group is preferred. In a preferred embodiment, both R^1 and R^2 are hydrogen.

[0163] Turning now to Y^1 and Y^2 , taken together, Y^1 and Y^2 may form $-O-C(O)-$, $O-C(S)-$, $S-C(S)-$, and $S-C(O)-$. In one or more preferred embodiments, Y^1 is oxygen and Y^2 is oxygen.

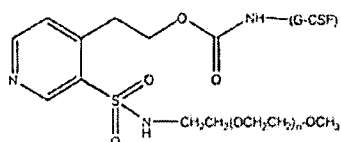
[0164] Optional electron-altering substituents on the fluorenyl ring include R^{e1} and/or R^{e2} . When present, R^{e1} and/or R^{e2} may be either an electron-withdrawing group or an electron-donating group. If both present, R^{e1} and R^{e2} may both be electron-withdrawing groups, electron-donating groups, or may comprise one electron donating group and one electron withdrawing group. Exemplary electron withdrawing groups include halogen, $-C(O)H$, $-C(O)R$, $-C(O)OR$, $-C(O)OH$, $-C(O)X$, where X is a halo group, $-CF_3$, $-C\equiv N$, and $-SO_3H$, where R is an organic radical. In a preferred embodiment, R^{e1} is $-SO_3H$. Exemplary electron donating groups include $-NH_2$, $-NRH$, $-NR_2$, $-OH$, $-OR$, and $-NHC(O)R$, where R is an organic radical.

[0165] Representative G-CSF conjugates have the following structures:





; and



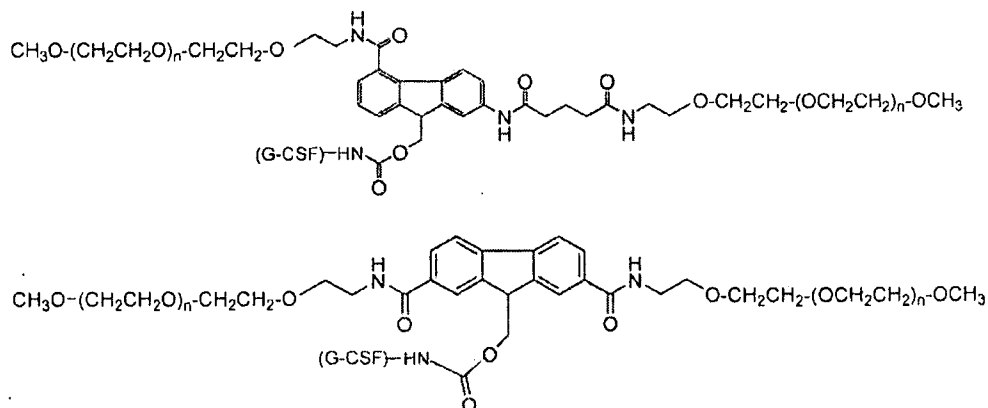
wherein, for each structure and in each instance, (n) is independently an integer from 4 to 1500, and (G-CSF) is a residue of a G-CSF moiety. The above structures are meant to encompass G-CSF conjugates having one or more polymeric reagents covalently attached to one or more amino residues within the G-CSF moiety.

[0166] Representative molecular weights for the polymer-portion of each of the above conjugates include the following: about 1,000 Daltons, about 1,500 Daltons, about 2,000 Daltons, about 2,200 Daltons, about 2,500 Daltons, about 3,000 Daltons, about 4,000 Daltons, about 4,400 Daltons, about 4,500 Daltons, about 5,000 Daltons, about 5,500 Daltons, about 6,000 Daltons, about 7,000 Daltons, about 7,500 Daltons, about 8,000 Daltons, about 9,000 Daltons, about 10,000 Daltons, about 11,000 Daltons, about 12,000 Daltons, about 13,000 Daltons, about 14,000 Daltons, about 15,000 Daltons, about 16,000 Daltons, about 17,000 Daltons, about 18,000 Daltons, about 19,000 Daltons, about 20,000 Daltons, about 22,500 Daltons, about 25,000 Daltons, about 30,000 Daltons, about 35,000 Daltons, about 40,000 Daltons, about 45,000 Daltons, about 50,000 Daltons, about 55,000 Daltons, about 60,000 Daltons, about 65,000 Daltons, about 70,000 Daltons, and about 75,000 Daltons. Preferred conjugates possess a polymer molecular weight of about 20,000 Daltons, 30,000 Daltons, 40,000 Daltons and 50,000 Daltons.

[0167] The amine to which the polymeric reagent couples can be at the N-terminus or an amine-containing side chain of an amino acid (such as lysine) within the G-CSF moiety. For recombinant G-CSF, sites suitable for attachment include the N-terminus and lysines 17, 24, 35, and 41. Due to the releasable nature of the conjugates, covalent attachment of a polymeric reagent to a lysine involved in receptor binding, e.g., lysines 17 and 24, is not expected to significantly impair the performance of the conjugate.

[0168] G-CSF releasable conjugates having a structure as provided herein include mono-conjugates (having polymeric reagent releasably attached to a single amino-site within the G-CSF moiety), di-conjugates (having polymeric reagent releasably attached to two different amino-sites within the G-CSF moiety), and tri-conjugates (having polymeric reagent releasably attached to a three different amino-sites within the G-CSF moiety), as well as mixtures of the foregoing. Preferred are G-CSF mono-conjugates having a releasable polymeric reagent covalently attached to a single site within the G-CSF moiety. Exemplary compositions are those substantially comprising a single G-CSF mono-conjugate - i.e., substantially comprising a single positional isomer where substantially all of the conjugates within the composition have the polymeric reagent attached to the same site within the G-CSF molecule. Illustrative conjugates and compositions are described in detail in the accompanying examples. Specifically, the preparation of both an unsymmetrical, releasable branched fluorenyl-based G-CSF conjugate and a symmetrical, releasable branched fluorenyl-based G-CSF conjugate is described in Example 1

(a-d) and Example 2 (a-d), respectively. The illustrative conjugates possess the structures shown below:



[0169] The conjugates prepared included mono-, di-, and tri-G-CSF conjugates. These conjugates were then subjected to additional separation/purification steps to provide G-CSF compositions substantially comprising a single mono-G-CSF conjugate in accordance with the foregoing structures; these compositions were then further purified to provide compositions substantially comprising a single positional isomer of a G-CSF mono-conjugate, as well as a mixture comprising each of the mono-conjugate positional isomers. Once having the positional isomer compositions in hand, one may, if desired, form customized mixtures of the individual positional isomers having varying relative amounts of each, based upon their individual release rates and activities, to provide a composition having a particular release profile.

Preparing the Conjugates

[0170] Conjugates in accordance with the invention are generally prepared by contacting a polymeric reagent as described herein with a G-CSF moiety under conditions suitable to form a releasable covalent attachment between the polymer and the G-CSF moiety. Typically, the polymer reagent is added to the G-CSF or G-CSF-containing surface at an equimolar amount (with respect to the desired number of groups suitable for reaction with the reactive group) or at a molar excess. For example, the polymeric reagent can be added to the G-CSF at a molar ratio of about 1:1 (polymeric reagent:G-CSF), 1.5:1, 2:1, 3:1, 4:1, 5:1, 6:1, 8:1, or 10:1. The conjugation reaction is allowed to proceed until substantially no further conjugation occurs, which can generally be determined by monitoring the progress of the reaction over time.

[0171] Examples 1a-1d and Examples 2a-2d describe illustrative methods for preparing G-CSF conjugates as described herein. Generally, an excess of a releasable PEG reagent (e.g., dissolved in acid) is added to a solution of the G-CSF moiety dissolved in buffer. A suitable pH range for the buffer is a pH of about 7-9. Should selective attachment at the N-terminus be desired, the reaction is then carried out at lower pHs, e.g., at around a pH of 5 or so. The solutions are then mixed, and allowed to couple. Generally, the reaction is allowed to proceed at room temperature, optionally followed by continued reaction under cooled temperatures, e.g., from about 0°C to about 20°C. For example, the reactants may initially be mixed at room temperature, e.g., 25°C, followed by reaction at a lower temperature such as 4°C. Generally the reaction will progress from minutes to several hours depending upon the reaction conditions employed. The reaction can be quenched, e.g., addition of acid to lower the pH.

[0172] Progress of the reaction can be monitored by withdrawing aliquots from the reaction mixture at various time points and analyzing the reaction mixture by SDS-PAGE or MALDI-TOF mass spectrometry or any other suitable analytical method. Once a plateau is reached with respect to the amount of conjugate formed or the amount of unconjugated polymer reagent remaining, the reaction is assumed to be complete. Typically, the conjugation reaction takes anywhere from minutes to several hours (e.g., from 5 minutes to 24 hours or more). The resulting product mixture is preferably, but not necessarily, purified to separate out excess reagents, unconjugated reactants (e.g., G-CSF) undesired multi-conjugated species, and free or unreacted polymer. The resulting conjugates can then be further characterized using analytical methods such as MALDI, capillary electrophoresis, gel electrophoresis, and/or various types of chromatography.

[0173] It is possible to characterize the degree of attachment (that is, the number -- often expressed in terms of an average number in the context of a composition of conjugates -- of polymeric reagents that are attached to the G-CSF) of the conjugate. To determine the average number of water-soluble polymer molecules within a given G-CSF conjugate, analytical techniques such as SDS-PAGE, SEC, IEC, MALDI-TOF, and so forth can be used. Spectrophotometric detection of the residual primary amines on G-CSF can also be utilized, e.g., using a trinitrobenzene sulfonic acid assay (TNBSA), to provide a qualitative and quantitative estimation of degree of polymer attachment. In Examples 1a-d and 2a-d, the resulting releasable conjugate fractions were purified by cation exchange chromatography, and the samples

collected. The G-CSF-water-soluble polymer conjugates formed were mostly mono- and di-conjugates.

[0174] With respect to polymer-G-CSF conjugates, the conjugates can be purified to obtain/isolate different conjugated species. Alternatively, and more preferably for lower molecular weight (e.g., less than about 20 kiloDaltons, more preferably less than about 10 kiloDaltons) polymers, the product mixture can be purified to obtain the distribution of water-soluble polymer segments per active agent. For example, the product mixture can be purified to obtain an average of anywhere from one to five polymeric reagents per G-CSF moiety. The strategy for purification of the final conjugate reaction mixture will depend upon a number of factors, including, for example, the molecular weight of the particular polymer employed, the particular G-CSF moiety employed, the desired dosing regimen, and the residual activity and *in vivo* properties of the individual conjugate(s).

[0175] If desired, conjugates having different molecular weights can be isolated using gel filtration chromatography. That is to say, gel filtration chromatography is used to fractionate differently numbered polymer-to-G-CSF ratios (e.g., 1-mer, 2-mer, 3-mer, and so forth, wherein "1-mer" indicates 1 polymer to G-CSF, "2-mer" indicates two polymers to G-CSF, and so on) on the basis of their differing molecular weights (where the difference corresponds essentially to the average molecular weight of the water-soluble polymer segments). For example, in an exemplary reaction where a 100 kDa protein is randomly conjugated to a polymeric reagent having a molecular weight of about 20 kDa, the resulting reaction mixture will likely contain unmodified protein (MW 100 kDa), mono-PEGylated protein (MW 120 kDa), di-PEGylated protein (MW 140 kDa), and so forth. While this approach can be used to separate PEG and other polymer conjugates having different molecular weights, this approach is generally ineffective for separating positional isomers having different polymer attachment sites within a protein such as G-CSF. For example, gel filtration chromatography can be used to separate from each other mixtures of PEG 1-mers, 2-mers, 3-mers, and so forth, although each of the recovered PEG-mer compositions may contain PEGs (or any other suitable water-soluble polymer) attached to different reactive amino groups (e.g., lysine residues) within the active agent.

[0176] Gel filtration columns suitable for carrying out this type of separation include Superdex™ and Sephadex™ columns available from Amersham Biosciences (Piscataway, NJ). Selection of a particular column will depend upon the desired fractionation range desired. Elution is generally carried out using a suitable buffer, such as phosphate, acetate, or the like.

The collected fractions may be analyzed by a number of different methods, for example, (i) optical density (OD) at 280 nm for protein content, (ii) bovine serum albumin (BSA) protein analysis, (iii) iodine testing for PEG content [Sims *et al.* (1980) *Anal. Biochem.*, 107:60-63], and (iv) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), followed by staining with barium iodide.

[0177] Separation of positional isomers is carried out by reverse phase chromatography using a reverse phase-high performance liquid chromatography (RP-HPLC) C18 column (Amersham Biosciences or Vydac) or by ion exchange chromatography using an ion exchange column, e.g., a Sepharose™ ion exchange column available from Amersham Biosciences. Either approach can be used to separate polymer-active agent isomers having the same molecular weight (positional isomers). Exemplary compositions comprising positional isomers and mixtures of positional isomers of G-CSF (e.g., mono-conjugates) are described in detail in the accompanying examples.

G-CSF Moiety

[0178] As stated above, an amine-containing biologically active agent for use in coupling to a polymer as presented herein is a G-CSF moiety.

[0179] The term "G-CSF moiety," as used herein, refers to a moiety having G-CSF activity, and, unless the context clearly dictates otherwise, also refers to a G-CSF precursor moiety (an exemplary sequence of which is provided in SEQ ID NO: 3). The G-CSF moiety will also have at least one electrophilic group or nucleophilic group suitable for reaction with a polymeric reagent. In addition, the term "G-CSF moiety" encompasses both the G-CSF moiety prior to conjugation as well as the G-CSF moiety residue following conjugation. As will be explained in further detail below, one of ordinary skill in the art can determine whether any given moiety has G-CSF activity. Proteins comprising an amino acid sequence corresponding to any one of SEQ ID NOS: 1 through 2 corresponds to a G-CSF moiety, as well as any protein or polypeptide substantially homologous thereto, whose biological properties result in the stimulation of growth and/or number of neutrophils and/or activity similar to G-CSF.

[0180] As used herein, the term "G-CSF moiety" includes such proteins modified deliberately, as for example, by site directed mutagenesis or accidentally through mutations. These terms also include analogs having from 1 to 6 additional glycosylation sites, analogs

having at least one additional amino acid at the carboxy terminal end of the protein wherein the additional amino acid(s) includes at least one glycosylation site, and analogs having an amino acid sequence which includes at least one glycosylation site. These terms include both natural and recombinantly produced G-CSF.

[0181] Human granulocyte-colony stimulating factor (G-CSF) is a member of the four-helix bundle family of cytokines. Endogenous G-CSF is a lineage specific colony-stimulating factor which is produced by monocytes, fibroblasts, and endothelial cells. G-CSF regulates the production of neutrophils within the bone marrow and affects neutrophil progenitor proliferation, differentiation, and selected end-cell functional activation (including enhanced phagocytic ability, priming of the cellular metabolism associated with respiratory burst, antibody dependent killing, and the increased expression of some functions associated with cell surface antigens).

[0182] Recombinant G-CSF is a 18.8 kDa protein containing 175 amino acid residues, and contains four lysines at positions 17, 24, 35, and 41, plus the N-terminus. Filgrastim is the name for recombinant human G-CSF, often designated r-metHuG-CSF. Commercially available rhG-CSF is manufactured by Amgen under the name, Neupogen®. Neupogen® is a 175 amino acid protein manufactured by recombinant DNA technology. Neupogen® is produced by *Escherichia coli* (*E coli*) bacteria into which has been inserted the human granulocyte colony-stimulating factor gene. NEUPOGEN® has a molecular weight of 18,800 daltons. The protein has an amino acid sequence that is identical to the natural sequence predicted from human DNA sequence analysis, except for the addition of an N-terminal methionine necessary for expression in *E coli*. Since NEUPOGEN® is produced in *E coli*, the product is nonglycosylated and thus differs from G-CSF isolated from a human cell. Glycosylated recombinant G-CSF is also referred to a lenograstim.

[0183] As used herein, the term "G-CSF moiety" shall refer to the G-CSF moiety prior to conjugation as well as to the G-CSF moiety following attachment to a nonpeptidic water-soluble polymer. It is understood, however, that when the G-CSF moiety is attached to a nonpeptidic water-soluble polymer, the G-CSF moiety is slightly altered due to the presence of one or more covalent bonds associated with linkage to the polymer. Often, this slightly altered form of the G-CSF moiety attached to another molecule is referred to a "residue" of the G-CSF moiety. The G-CSF moiety in the conjugate can be any moiety that provides a granulocyte-colony stimulating factor effect.

[0184] The G-CSF moiety can be derived from either non-recombinant methods or from recombinant methods and the invention is not limited in this regard. In addition, the G-CSF moiety can be derived from human sources or from animal sources.

[0185] The G-CSF moiety can be derived non-recombinantly. For example, as described in U.S. Patent No. 4,810,643, one may collect G-CSF from the culture medium of a human carcinoma cell line denominated 5637 and deposited under restrictive conditions with the American Type Culture Collection, Rockville MD as A.T.C.C. Deposit No. HTB-9.

[0186] The G-CSF moiety can be derived from recombinant methods and can be expressed in bacterial (e.g., *E. coli*), mammalian (e.g., Chinese hamster ovary cells), and/or yeast (e.g., *Saccharomyces cerevisiae*) expression systems. The expression can occur via exogenous expression or via endogenous expression. For example, Nagata et al. (1986), *Nature* 319:415 provides the cDNA for human G-CSF ("hG-CSF") isolated from human squamous cell carcinoma cell line CHU-II and also describes a process for expressing of the protein in COS cells (African Green Monkey cells). Souza et al. describes a process for expressing G-CSF in *E. coli* cells. U.S. Patent No. 4,810,643 describes recombinant-based methods for preparing methionyl G-CSF (i.e., G-CSF to which the N-terminus has the amino acid methionine attached). In addition, U.S. Patent No. 5,633,352 describes recombinant methods for preparing G-CSF.

[0187] The amino acid sequence for human G-CSF is provided in SEQ ID NO: 1. As provided therein, a methionine residue-containing form (wherein $n''' = 1$) is also contemplated for this, and all other sequences, described herein. SEQ ID NO 2 corresponds to G-CSF moiety having a different sequence than SEQ ID NO 1.

[0188] Although recombinant-based methods for preparing proteins can differ, recombinant methods typically involve constructing the nucleic acid encoding the desired polypeptide or fragment, cloning the nucleic acid into an expression vector, transforming a host cell (e.g., plant, bacteria, yeast, transgenic animal cell, or mammalian cell such as Chinese hamster ovary cell or baby hamster kidney cell), and expressing the nucleic acid to produce the desired polypeptide or fragment. Methods for producing and expressing recombinant polypeptides *in vitro* and in prokaryotic and eukaryotic host cells are known to those of ordinary skill in the art.

[0189] To facilitate identification and purification of the recombinant polypeptide, nucleic acid sequences that encode for an epitope tag or other affinity binding sequence can be

inserted or added in-frame with the coding sequence, thereby producing a fusion protein comprised of the desired polypeptide and a polypeptide suited for binding. Fusion proteins can be identified and purified by first running a mixture containing the fusion protein through an affinity column bearing binding moieties (e.g., antibodies) directed against the epitope tag or other binding sequence in the fusion proteins, thereby binding the fusion protein within the column. Thereafter, the fusion protein can be recovered by washing the column with the appropriate solution (e.g., acid) to release the bound fusion protein. The recombinant polypeptide can also be identified and purified by lysing the host cells, separating the polypeptide, e.g., by size exclusion chromatography, and collecting the polypeptide. These and other methods for identifying and purifying recombinant polypeptides are known to those of ordinary skill in the art. In one or more embodiments of the invention, however, it is preferred that the G-CSF moiety is not in the form of a fusion protein.

[0190] Depending on the system used to express proteins having G-CSF activity, the G-CSF moiety can be unglycosylated or glycosylated and either may be used. That is, the G-CSF moiety can be unglycosylated or the G-CSF moiety can be glycosylated. In one or more embodiments of the invention, it is preferred that the G-CSF moiety is not glycosylated.

[0191] The G-CSF moiety can advantageously be modified to include one or more amino acid residues such as, for example, lysine, cysteine and/or arginine, in order to provide facile attachment of a polymer to an atom within the side chain of the amino acid. In addition, the G-CSF moiety can be modified to include a non-naturally occurring amino acid residue. Techniques for adding amino acid residues and non-naturally occurring amino acid residues are well known to those of ordinary skill in the art. Reference is made to J. March, *Advanced Organic Chemistry: Reactions Mechanisms and Structure*, 4th Ed. (New York: Wiley-Interscience, 1992). In one or more embodiments of the invention, it is preferred that the G-CSF moiety is not modified to include one or more amino acid residues. Exemplary G-CSF moieties having at least one substitution relative to hG-CSF are provided in U.S. Patent No. 6,646,110, and are suited for use as a G-CSF moiety herein. Further, exemplary G-CSF moieties having at least one substitution relative to hG-CSF are provided in U.S. Patent Nos. 6,004,548 and 5,580,755, and are suited for use as a G-CSF moiety herein.

[0192] In addition, the G-CSF moiety can advantageously be modified to include attachment of a functional group (other than through addition of a functional group-containing amino acid residue). For example, the G-CSF moiety can be modified to include a thiol group.

In addition, the G-CSF moiety can be modified to include an N-terminal alpha carbon. In addition, the G-CSF moiety can be modified to include one or more carbohydrate moieties. In some embodiments of the invention, it is preferred that the G-CSF moiety is not modified to include a thiol group and/or an N-terminal alpha carbon. G-CSF moieties containing an aminoxy, aldehyde or some other functional group can be used.

[0193] A preferred G-CSF moiety has an amino acid sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2. Unless specifically noted, all assignments of a numeric location of an amino acid residue as provided herein are based on SEQ ID NO: 1 (ignoring any leading methionyl residue). Sequences that are useful to serve as G-CSF moieties include those sequences of the proteins found in commercially available versions of G-CSF-containing formulations such as NEUPOGEN® G-CSF (Amgen, Thousand Oaks, CA) and GRASTIM® G-CSF (Dr. Reddy's, Hyderabad, India).

[0194] hG-CSF moiety (as provided in SEQ ID NO: 1) can be used as well as truncated versions, hybrid variants, and peptide mimetics of the sequence. Biologically active fragments, deletion variants, substitution variants or addition variants of any of the foregoing that maintain at least some degree of G-CSF activity can also serve as a G-CSF moiety.

[0195] For any given peptide or protein moiety, it is possible to determine whether that moiety has G-CSF activity. For example, as described in U.S. Patent No. 5,580,755, it is possible to administer a G-CSF moiety of interest with buffer into the blood stream of a hamster and count the granulocytes. The G-CSF moiety of interest can serve as an G-CSF moiety in accordance with the present invention if the hamster injected with the proposed G-CSF moiety exhibits a statistically significant increase in granulocytes when compared to a control hamster not injected with the proposed G-CSF moiety (e.g., simply buffer).

Pharmaceutical Compositions

[0196] The present invention also includes pharmaceutical preparations comprising a conjugate as provided herein in combination with a pharmaceutical excipient or carrier. Generally, the conjugate itself will be in a solid form (e.g., a precipitate), which can be combined with a suitable pharmaceutical excipient that can be in either solid or liquid form.

[0197] Exemplary excipients comprised within a pharmaceutical composition may include, without limitation, those selected from the group consisting of carbohydrates, inorganic

salts, antimicrobial agents, antioxidants, surfactants, buffers, acids, bases, and combinations thereof.

[0198] A carbohydrate such as a sugar, a derivatized sugar such as an alditol, aldonic acid, an esterified sugar, and/or a sugar polymer may be present as an excipient. Specific carbohydrate excipients include, for example: monosaccharides, such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol, sorbitol (glucitol), pyranosyl sorbitol, myoinositol, and the like.

[0199] The excipient can also include an inorganic salt or buffer such as citric acid, sodium chloride, potassium chloride, sodium sulfate, potassium nitrate, sodium phosphate monobasic, sodium phosphate dibasic, and combinations thereof.

[0200] The preparation may also include an antimicrobial agent for preventing or deterring microbial growth. Nonlimiting examples of antimicrobial agents suitable for the present invention include benzalkonium chloride, benzethonium chloride, benzyl alcohol, cetylpyridinium chloride, chlorobutanol, phenol, phenylethyl alcohol, phenylmercuric nitrate, thimersol, and combinations thereof.

[0201] An antioxidant can be present in the preparation as well. Antioxidants are used to prevent oxidation, thereby preventing the deterioration of the conjugate or other components of the preparation. Suitable antioxidants for use in the present invention include, for example, ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, hypophosphorous acid, monothioglycerol, propyl gallate, sodium bisulfite, sodium formaldehyde sulfoxylate, sodium metabisulfite, and combinations thereof.

[0202] A surfactant may be present as an excipient. Exemplary surfactants include: polysorbates, such as "Tween 20" and "Tween 80," and pluronics such as F68 and F88 (both of which are available from BASF, Mount Olive, New Jersey); sorbitan esters; lipids, such as phospholipids such as lecithin and other phosphatidylcholines, phosphatidylethanolamines (although preferably not in liposomal form), fatty acids and fatty esters; steroids, such as cholesterol; and chelating agents, such as EDTA, zinc and other such suitable cations.

[0203] Acids or bases may be present as an excipient in the preparation. Nonlimiting examples of acids that can be used include those acids selected from the group consisting of

hydrochloric acid, acetic acid, phosphoric acid, citric acid, malic acid, lactic acid, formic acid, trichloroacetic acid, nitric acid, perchloric acid, phosphoric acid, sulfuric acid, fumaric acid, and combinations thereof. Examples of suitable bases include, without limitation, bases selected from the group consisting of sodium hydroxide, sodium acetate, ammonium hydroxide, potassium hydroxide, ammonium acetate, potassium acetate, sodium phosphate, potassium phosphate, sodium citrate, sodium formate, sodium sulfate, potassium sulfate, potassium fumarate, and combinations thereof.

[0204] The pharmaceutical preparations encompass all types of formulations and in particular those that are suited for injection, e.g., powders that can be reconstituted as well as suspensions and solutions. The amount of the releasable G-CSF conjugate in the composition will vary depending on a number of factors, but will optimally be a therapeutically effective dose when the composition is stored in a unit dose container (e.g., a vial). In addition, the pharmaceutical preparation can be housed in a syringe. A therapeutically effective dose can be determined experimentally by repeated administration of increasing amounts of the conjugate in order to determine which amount produces a clinically desired endpoint.

[0205] The amount of any individual excipient in the composition will vary depending on the activity of the excipient and particular needs of the composition. Typically, the optimal amount of any individual excipient is determined through routine experimentation, i.e., by preparing compositions containing varying amounts of the excipient (ranging from low to high), examining the stability and other parameters, and then determining the range at which optimal performance is attained with no significant adverse effects.

[0206] Generally, however, the excipient will be present in the composition in an amount of about 1% to about 99% by weight, preferably from about 5%-98% by weight, more preferably from about 15-95% by weight of the excipient, with concentrations less than 30% by weight most preferred.

[0207] An exemplary composition will have a pH of from 3.0 to 5.0 (e.g., a pH of 4.0). With respect to components, an exemplary composition will have a 0.1% w/v to 10% w/v (e.g., 1% w/v) of conjugate, 0.0058% w/v to 0.58% w/v (e.g., 0.058% wv) of acetate, 0.5% w/v to 50% w/v (e.g., 5% w/v) of sorbitol, 0.00033% w/v to 0.033% w/v (e.g., 0.0033% w/v) of polysorbate 20, and 0.00035% w/v to 0.035% w/v (e.g., 0.0035% w/v) of sodium.

[0208] These foregoing pharmaceutical excipients along with other excipients are described in "Remington: The Science & Practice of Pharmacy", 19th ed., Williams & Williams, (1995), the "Physician's Desk Reference", 52nd ed., Medical Economics, Montvale, NJ (1998), and Kibbe, A.H., Handbook of Pharmaceutical Excipients, 3rd Edition, American Pharmaceutical Association, Washington, D.C., 2000.

Administration

[0209] The pharmaceutical preparations of the present invention are typically, although not necessarily, administered via injection and are therefore generally liquid solutions or suspensions immediately prior to administration. The pharmaceutical preparation can also take other forms such as syrups, creams, ointments, tablets, powders, and the like. Other modes of administration are also included, such as pulmonary, rectal, transdermal, transmucosal, oral, intrathecal, subcutaneous, intra-arterial, and so forth.

[0210] As previously described, the releasable G-CSF conjugates can be administered parenterally by intravenous injection, or less preferably by intramuscular or by subcutaneous injection. Suitable formulation types for parenteral administration include ready-for-injection solutions, dry powders for combination with a solvent prior to use, suspensions ready for injection, dry insoluble compositions for combination with a vehicle prior to use, and emulsions and liquid concentrates for dilution prior to administration, among others.

[0211] Also provided herein is a method for administering a releasable G-CSF conjugate as provided herein to a patient suffering from a condition that is responsive to treatment with conjugate such as neutropenia. Generally, a releasable G-CSF conjugate composition as described herein may be administered to a patient undergoing certain types of cancer treatment, to boost the patient's white blood cell count, i.e., for the treatment of neutropenia. A releasable G-CSF conjugate composition as provided herein may be administered to treat neutropenia resulting from any of a number of causes, such as bone marrow transplant, HIV, drug-induced neutropenia, and the like. The method comprises administering, generally via injection, a therapeutically effective amount of the releasable G-CSF conjugate (preferably provided as part of a pharmaceutical preparation). The method of administering may be used to treat any condition that can be remedied or prevented by administration of the G-CSF moiety. For example, the releasable G-CSF conjugates provided herein can suitably be administered to

patients with most types of cancer undergoing moderately myelosuppressive chemotherapy to help protect the patients from infection which can result from low white blood cell counts.

[0212] Those of ordinary skill in the art appreciate which conditions a specific conjugate can effectively treat. The actual dose to be administered will vary depend upon the age, weight, and general condition of the subject as well as the severity of the condition being treated, the judgment of the health care professional, and conjugate being administered. Therapeutically effective amounts are known or may be readily determined by those skilled in the art and/or are described in the pertinent reference texts and literature. Generally, a therapeutically effective amount will range from about 0.001 mg to 100 mg, preferably in doses from 0.01 mg/day to 75 mg/day, and more preferably in doses from 0.10 mg/day to 50 mg/day. For instance, a therapeutically effective amount of a releasable G-CSF conjugate composition as provided herein may range from an amount that averages to about 0.50 microgram/kg/day to about 20 microgram/kg/day, e.g., may be about 0.50 microgram/kg/day, 1.0 microgram/kg/day, 2.0 microgram/kg/day, 3.0 microgram/kg/day, 4.0 microgram/kg/day, 5.0 microgram/kg/day, 6.0 microgram/kg/day, 7.0 microgram/kg/day, 8.0 microgram/kg/day, 9.0 microgram/kg/day, 10.0 microgram/kg/day, 11.0 microgram/kg/day, 12 microgram/kg/day, 13 microgram/kg/day, 14 microgram/kg/day, 15 microgram/kg/day, 16 microgram/kg/day, 17 microgram/kg/day, 18 microgram/kg/day, 19 microgram/kg/day, or 20 microgram/kg/day, depending upon the factors noted above, although the actual dosing regimen is not necessarily daily.

[0213] The unit dosage of any given conjugate (again, preferably provided as part of a pharmaceutical preparation) can be administered in a variety of dosing schedules depending on the judgment of the clinician, needs of the patient, and so forth. The specific dosing schedule will be known by those of ordinary skill in the art or can be determined experimentally using routine methods. Exemplary dosing schedules include, without limitation, administration five times a day, four times a day, three times a day, twice daily, once daily, three times weekly, twice weekly, once weekly, twice monthly, once monthly, and any combination thereof. For example, dosing may commence at a certain time point following a round of chemotherapy, e.g., within 24 hours, within 48 hours, within 36 hours, and so on. Once the clinical endpoint has been achieved, dosing of the composition is halted.

[0214] It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, that the foregoing description as well as the experimental that follow are intended to illustrate and not limit the scope of the invention. Other

aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

[0215] All articles, books, patents, patent publications and other publications referenced herein are hereby incorporated by reference in their entireties.

EXAMPLES

[0216] The practice of the invention will employ, unless otherwise indicated, conventional techniques of organic synthesis and the like, which are understood by one of ordinary skill in the art. Such techniques are fully explained in the literature. See, for example, J. March, *Advanced Organic Chemistry: Reactions Mechanisms and Structure*, 6th Ed. (New York): Wiley-Interscience, 2007), *supra*.

[0217] In the following examples, efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, and so forth), but some experimental error and deviation should be accounted for. Unless otherwise indicated, temperature is in degrees Celsius and pressure is at or near atmospheric pressure at sea level. Each of the following examples is considered to be instructive to one of ordinary skill in the art for carrying out one or more embodiments described herein.

Materials and Methods

[0218] Recombinant human G-CSF ("rhG-CSF") used in the examples was obtained from *E. coli*.

[0219] SDS-PAGE Analysis: Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an Invitrogen gel electrophoresis system (XCell SureLock Mini-Cell). Samples were mixed with sample buffer. Then, the prepared samples were loaded onto a gel and run for approximately thirty minutes.

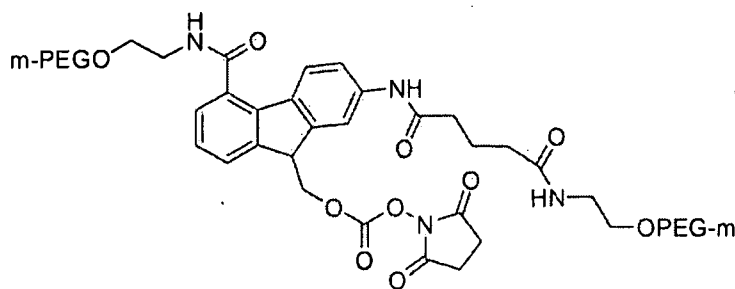
[0220] SEC-HPLC Analysis: Size exclusion chromatography (SEC-HPLC) analysis was performed on an Agilent 1100 HPLC system (Agilent). Samples were analyzed using a TSK-GEL G3000SWxl column (7.8 x 300 mm, Phenomenex), and a mobile phase consisting of 100 mM phosphoric acid, pH 2.5. The flow rate for the column was 0.5 ml/min. Eluted protein and PEG-protein conjugates were detected using UV at 227 nm and 280 nm.

[0221] RP-HPLC Analysis: Reversed phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 HPLC system (Agilent). Samples were analyzed using a Zorbax 300SB-C3 column (3.5 μ m particle size, 150 mm x 3.0 mm, Agilent), and mobile phases consisting of 0.1% trifluoroacetic acid in water (buffer A) and 0.1% trifluoroacetic acid in acetonitrile (buffer B). The flow rate for the column was 0.3 ml/min. The protein and PEG-protein conjugates were eluted with a linear gradient over 25 minutes, and were detected using UV at 280nm.

[0222] Cation Exchange Chromatography: A HiTrap SP Sepharose HP cation exchange column (GE Healthcare, Piscataway NJ) was used with the AKTA purification system (GE Healthcare, Piscataway NJ) to purify the PEG-G-CSF conjugates prepared. For each conjugate solution prepared, the conjugate solution was loaded on a column that was pre-equilibrated in 20 mM sodium acetate buffer, pH 4.0 (buffer A) and then washed with a few column volumes of buffer A to remove any unreacted PEG reagent. Subsequently, a gradient of buffer A with 0-100% buffer B (20mM NaOAc with 0.5 M NaCl buffer, pH 4.0) was raised. The eluent was monitored by UV detector at 280 nm. The di-PEG-G-CSF conjugates were eluted first, followed by mono-PEG-G-CSF positional isomers, and finally the unconjugated G-CSF. The fractions were pooled according to the chromatogram, and the purity of the individual conjugate was determined by HPLC or SDS-PAGE.

Examples 1a, 1b, 1c and 1d

Preparation of Mono-Conjugates: "CG-FMOC-PEG2-40K-GCSF-A," "CG-FMOC-PEG2-40K-GCSF-B," "CG-FMOC-PEG2-40K-GCSF-C" and "CG-FMOC-PEG2-40K-GCSF-M" Using 9-Hydroxymethyl-[4-carboxamido M-PEG (20,000)-7-amidoglutaric amide M-PEG(20,000)]fluorene-N-Hydroxysuccinimide Derivative, 40kDa "CG-FMOC-PEG2-40K-NHS

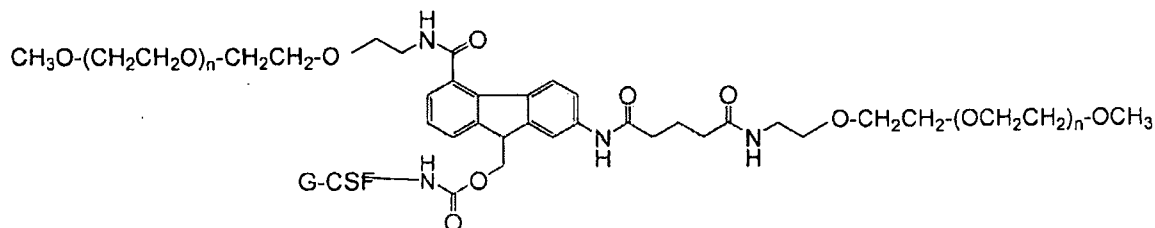


PEG Reagent Employed: "CG-FMOC-PEG2-40K-NHS," 40 kDa (PEG-m indicates methoxy PEG or mPEG)

[0223] CG-FMOC-PEG2-40K-NHS, 40 kDa, stored at -20°C under argon, was warmed to ambient temperature. An excess (relative to the amount of G-CSF in a measured aliquot of the stock G-CSF solution) of the warmed CG-FMOC-PEG2-40K-NHS was dissolved in 2 mM HCl to form a 10% reagent solution. The 10% reagent solution was quickly added to the aliquot of stock G-CSF solution (4 mg/ml buffer solution, pH 7-9) and mixed well. To allow for coupling of the CG-FMOC-PEG2-40K-NHS to G-CSF via a carbamate linkage, the reaction solution was maintained at room temperature first, and then at 4°C . The reaction was quenched by the addition of acetic acid to lower the pH to 4.0. The conjugate solution was characterized by SDS-PAGE and SEC-HPLC.

[0224] A cation-exchange chromatography method using SP Sepharose High Performance and NaOAc buffer was used to purify the conjugate solution such that mono-conjugates (i.e., the polymeric reagent attached at a single location to the G-CSF) were collected (and substantially lacking CG-FMOC-PEG2-40K-NHS, di-conjugates and unconjugated G-CSF). In this regard, FIG. 1 shows the chromatogram produced during cation-exchange chromatography. Collection of individual fractions was carried out such that four compositions resulted: a "mono-a" composition, a "mono-b" composition, a "mono-c" compositions, and a composition of each of "mono-a," mono-b, and "mono-c" (corresponding to "CG-FMOC-PEG2-40K-GCSF-A" (Example 1A), "CG-FMOC-PEG2-40K-GCSF-B" (Example 1B), "CG-FMOC-PEG2-40K-GCSF-C" (Example 1C) and "CG-FMOC-PEG2-40K-GCSF-M" (Example 1D), respectively) as indicated in FIG. 1. These compositions were then subjected to further analyses. Each of the compositions, CG-PEG-40K-GCSF-A", "CG-PEG-40K-GCSF-B", and "CG-PEG-40K-GCSF-C" corresponds to a mono-G-CSF conjugate as shown below, each representing a

different positional isomer (i.e., covalently attached to a different amino group/amino acid location) on the G-CSF molecule:

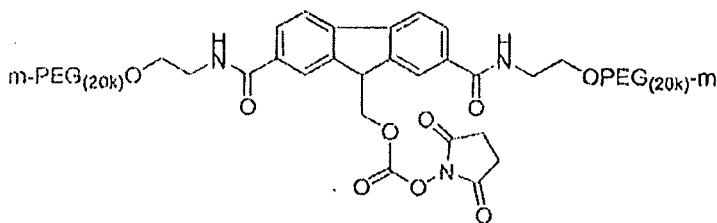


[0225] The "CG-FMOC-PEG2-40K-GCSF-M" composition represents a mixture of the mono-conjugate positional isomers, mono-a, mono-b, and mono-c, described above.

[0226] Using the synthetic approach described above and subsequent purification techniques, additional conjugates and conjugate compositions are prepared and purified using the CG-PEG2-FMOC-NHS reagent having other weight average molecular weights, e.g., CG-PEG2-FMOC-NHS, 10kDa, 20kDa, 30kDa, 50kDa, and so forth.

Examples 2a, 2b, 2c and 2d

Preparation of Preparation of Mono-Conjugates "C2-FMOC-PEG2-40K-GCSF-A," "C2-FMOC-PEG2-40K-GCSF-B," "C2-FMOC-PEG2-40K-GCSF-C" and "C2-FMOC-PEG2-40K-GCSF-M" Using "C2-FMOC-PEG2-NHS", 40 kDa



PEG Reagent Employed: "C2-FMOC-PEG2-40K-NHS," 40 kDa

[0227] C2- FMOC-PEG2-40K-NHS, 40kDa, a PEG reagent having a structure different from that employed in Examples 1a, 1b, 1c, and 1d, stored at -20°C under argon, was warmed to ambient temperature. An excess (relative to the amount of G-CSF in a measured aliquot of the stock G-CSF solution) of the warmed C2- FMOC-PEG2-40K-NHS was dissolved in 2 mM HCl to form a 10% reagent solution. The 10% reagent solution was quickly added to the aliquot of

stock G-CSF solution (4 mg/ml buffer solution, pH 7-9) and mixed well. To allow for coupling of the C2- FMOC-PEG2-40K-NHS to G-CSF via a carbamate linkage, the reaction solution was kept at room temperature first, and then at 4°C. The reaction was quenched by the addition of acetic acid to lower the pH to 4.0. The conjugate solution was characterized by SDS-PAGE, SEC-HPLC.

[0228] A cation-exchange chromatography method using SP Sepharose High Performance and NaOAc buffer was used to purify the conjugate solution such that mono-conjugates (i.e., the branched PEG reagent attached a single location on the G-CSF) were collected (and substantially lacking C2-FMOC-PEG2-40K-NHS, 40kDa, di-conjugates and unconjugated G-CSF). In this regard, FIG. 2 shows the chromatogram produced during cation-exchange chromatography. Individual fractions were collected such that four compositions resulted: a "mono-a" composition, a "mono-b" composition, a "mono-c" compositions, and a composition of each of "mono-a," mono-b, and "mono-c" (corresponding to "C2-FMOC-PEG2-40K-GCSF-A" (Example 2A), "C2-FMOC-PEG2-40K-GCSF-B" (Example 2B), "C2-FMOC-PEG2-40K-GCSF-C" (Example 2C) and "C2-FMOC-PEG2-40K-GCSF-M" (Example 2D), respectively) as indicated in FIG. 2. C2- FMOC-PEG2-40K-GCSF-A", "C2- FMOC-PEG2-40K-GCSF-B", and "C2-FMOC-PEG2-40K-GCSF-C" each represent different mono-G-CSF conjugate positional isomers having the PEG reagent covalently attached to a different location (amino acid amino group) within the G-CSF molecule. The mono-d composition contains a mixture of the three positional isomers in approximately equal amounts. These compositions were then subjected to further analyses.

[0229] Using the synthetic approach and purification techniques described above, additional conjugates and conjugate compositions are prepared and purified using C2-FMOC-PEG2-NHS reagents having other weight average molecular weights, e.g., C2-FMOC-PEG2-NHS, 10kDa, 20kDa, 30kDa, 50kDa, and so forth.

Example 3

Demonstration of the Releasable Nature of G-CSF Conjugates Under Physiological Conditions

[0230] Mono-G-CSF conjugates (mixtures of positional isomers), CG-FMOC-PEG2-40K-GCSF-M and C2-FMOC-PEG2-40K-GCSF-M (prepared as described in Examples 1a, 1b,

1c, 1d, 2a, 2b, 2c 2d) were demonstrated to possess a releasable linkage effective to liberate G-CSF *in vitro*.

[0231] Briefly, each mono-conjugate mixture was placed in a separate PBS buffer solution, pH 7.4, 37°C, *in vitro* and allowed to incubate for over 200 hours. Following incubation, aliquots from each were removed and tested for release of G-CSF using reverse phase HPLC. Release of G-CSF was detected in each mixture. An exemplary reverse phase HPLC chromatogram is provided as FIG. 3 for CG- FMOC-PEG2-40K-GCSF-M and as FIG. 4 for C2- FMOC-PEG2-40K-GCSF-M. As shown therein, following incubation, a mixture of released PEG, remaining mono-conjugate(s) and liberated G-CSF was detected, thereby confirming the ability of the mono-conjugates to release G-CSF.

[0232] While FIGS. 3 and 4 demonstrate the components of the G-CSF mono-conjugate solutions post-incubation, FIGS. 5A and 5B (CG-FMOC-PEG2-40K-GCSF-M), and 6A and 6B (C2- FMOC-PEG2-40K-GCSF-M) illustrate the release of G-CSF from the mono-conjugate mixtures over time. As shown in FIG. 5A, the release profile of CG- FMOC-PEG2-40K-GCSF-M, upon incubation at pH 7.4, 37°C, was expressed as the HPLC peak area of the conjugate remaining as a function of time. FIG. 5B is a linearized plot natural log plot based upon the same hydrolysis rate data. The “half life” of the CG-FMOC-PEG2-40K-GCSF-M mixture was calculated as 59 hours. See FIG. 5B. It is noted that the release kinetics were not completely linear overall when plotted as $\ln(A/A_0)$, consistent with the different release rates for different positional mono-isomers within the mixture. As can be discerned from the plots, the mono-conjugate mixture contains a mixture of faster and slower release mono-conjugates, thereby allowing one to tailor the release characteristics of a G-CSF mono-conjugate mixture by adjusting the relative amounts of the various mono-conjugates in the mixture.

[0233] The release kinetics of another exemplary G-CSF mono-conjugate mixture is shown in FIGS. 6A and 6B. As shown in FIG. 6A, the release profile of C2- FMOC-PEG2-40K-GCSF-M upon incubation at pH 7.4, 37°C was expressed as the HPLC peak area of the conjugate remaining as a function of time. As can be seen from a comparison of FIGS. 6A and 5A, the C2- FMOC-PEG2-40K-GCSF-M composition generally releases G-CSF more rapidly than does the CG- FMOC-PEG2-40K-GCSF-M mixture. Looking at FIG. 6A, it can be seen that essentially all of the G-CSF is liberated by 120 hours, while in comparison, residual conjugate is still detected at 200 hours for the CG- FMOC-PEG2-40K-GCSF-M composition. The “half life” of the C2-FMOC-PEG2-40K-GCSF-M was calculated as 25 hours from the linear

plot of the hydrolysis rate. See FIG. 6B. Again, it is noted that the release kinetics were not completely overall linear when plotted as $\ln(A/A_0)$, consistent with different release rates for different positional isomers contained within the mixture.

[0234] Using a similar approach to that described above, the release rates of individual mono-conjugates (prepared as described in Examples 1a, 1b, 1c, 1d, 2a, 2b, 2c 2d) were determined. **Table 1** shows the release rates of individual CG- FMOC-PEG2-40K-GCSF mono-conjugates (having polymer attached at different positions within the G-CSF molecule) and **Table 2** shows the release rates of individual C2- FMOC-PEG2-40K-GCSF mono-conjugates (having polymer attached at different positions within the G-CSF molecule). The data provided herein demonstrates a desirable slow continuous release of of G-CSF (in contrast to undesirable burst kinetics), thereby illustrating the therapeutic advantages of conjugates and compositions as provided herein.

[0235] As can be seen, by modifying the content of various CG- FMOC-PEG2-40K-GCSF mono-conjugates in a composition, one can tailor a composition having a particular G-CSF release profile. For instance, a therapeutic composition may comprise a mixture of CG- FMOC-PEG2-40K-GCSF-A, CG- FMOC-PEG2-40K-GCSF-B and CG- FMOC-PEG2-40K-GCSF-C, in any combination of relative amounts of each (tri-positional isomer mixture). Alternatively, a therapeutic composition may comprise a mixture of CG- FMOC-PEG2-40K-GCSF-A and CG- FMOC-PEG2-40K-GCSF-B, or CG-FMOC-PEG2-40K-GCSF-A and CG-FMOC-PEG2-40K-GCSF-C, or CG- FMOC-PEG2-40K-GCSF-B and CG-FMOC-PEG2-40K-GCSF-C in any combination of relative amounts of each component (di-positional isomer mixture). The foregoing similarly applies to C2-FMOC-PEG2-40K-GCSF- conjugate mixtures.

[0236] Alternatively, a composition comprising a single mono-conjugate positional isomer can be used to provide a therapeutic composition having a desired release rate of G-CSF, e.g., see the differences between conjugates A, B, and C below.

Table 1
Release Rates of Individual CG-FMOC-PEG2-40K-GCSF Mono-Conjugates

% Conjugate Remaining	16 Hours	92-98 Hours	139-140 Hours	155-156 Hours
CG-FMOC-PEG2-40K-GCSF-A	19%	0%	0%	0%
CG- FMOC-PEG2-40K-GCSF-B	30%	10%	6%	5%
CG-FMOC-PEG2-40K-GCSF-C	83%	30%	16%	15%

Table 2
Release Rates of Individual C2- FMOC-PEG2-40K-GCSF Mono-Conjugates

% Conjugate Remaining	16 Hours	32 Hours	56 Hours	80 Hours
C2- FMOC-PEG2-40K-GCSF-A	35%	14%	0%	0%
C2- FMOC-PEG2-40K-GCSF-B	30%	22%	13%	9%
C2- FMOC-PEG2-40K-GCSF-C	61%	36%	17%	8%

Example 4

In vitro Cell Proliferation Assays

[0237] *In vitro* cell proliferation assays in an M-NFS-60 cell line were performed to determine the relative activities of the three positional isomers of each conjugate series. Briefly, the M-NFS-60 (mouse myeloid leukemia) cell line responds to growth factors wherein activity is based on the stimulatory effect of a test article via presence of ATP, which signals the presence of metabolically active cells (Promega's CellTiter-Glo[®] kit). At the initiation of the study, about 4,000 cells (~90 μ L) and test article (in ~10 μ L) at eight different concentrations were each incubated at 37° C with 5% CO₂ conditions for 72 hours. At 72 hours, CellTiter-Glo[®] reagent (100 μ L) was added to each sample followed by mixing for two minutes. Following incubation for ten minutes at room temperature, proliferation was read with a luminescence counter for determination of EC50 values.

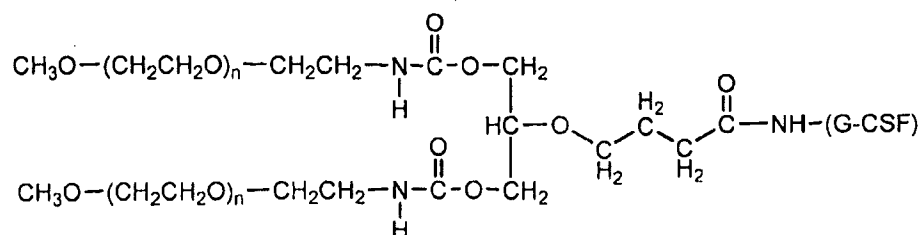
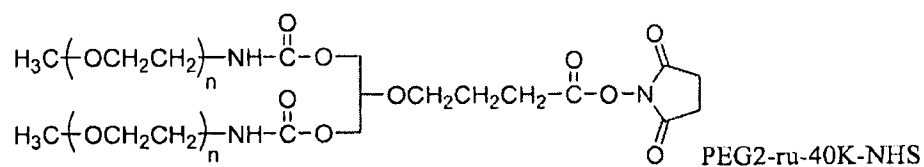
[0238] **Table 3** lists the potencies at 72 hours of the individual mono-conjugate positional isomers and mixtures prepared in connection with Examples 1a, 1b, 1c, 1d, 2a, 2b, 2c, and 2d compared to commercial pegfilgrastim (a conjugate of recombinant methionyl human G-CSF and monomethoxyPEG, 20kD, sold under the Neulasta[®] brand from Amgen Inc., Thousand Oaks, CA) or a rhG-CSF. Pegfilgrastim contains a linear PEG moiety stably (i.e., non-releasably) attached to G-CSF.

Table 3
Relative Potency of Tested G-CSF Preparations

Compound	EC50 (ng/mL)	Potency relative to pegfilgrastim (%)	Potency relative to G-CSF (%)
pegfilgrastim	0.02328	100	-
rhG-CSF	0.02515	93	100
C2-FMOC-PEG2-40K-GCSF-M	0.03164	74	79
CG-FMOC-PEG2-40K-GCSF-M	0.03309	70	76
C2-FMOC-PEG2-40K-GCSF-A	0.03027	77	83
C2-FMOC-PEG2-40K-GCSF-B	0.03509	66	72
C2- FMOC-PEG2-40K-GCSF-C	0.03228	72	78
CG- FMOC-PEG2-40K-GCSF-A	0.03502	66	72
CG-FMOC-PEG2-40K-GCSF-B	0.03437	68	73
CG-FMOC-PEG2-40K-GCSF-C	0.06143	38	41

[0239] Graphical representation of the same data is shown in FIG. 7. As can be seen, both of the mono-conjugate mixtures, C2-FMOC-PEG2-40K-GCSF-M and CG- FMOC-PEG2-40K-GCSF-M, possess good potencies (i.e., ability to proliferate M-NFS-60 cells) relative to both the commercial product, pegfilgrastim, as well as to G-CSF. Moreover, each of the mono-conjugate positional isomers demonstrates good potency in the *in-vitro* model employed, with all but CG- FMOC-PEG2-40K-GCSF-C demonstrating a potency in the *in-vitro* model of about 70% or greater relative to pegfilgrastim.

[0240] The stimulatory activity of the releasable PEG-GCSF conjugate compositions, C2-FMOC-PEG2-40K-GCSF-M and CG-FMOC-PEG2-40K-GCSF-M, was similarly measured over time and compared to the activity of a non-releasable conjugate composition comprising G-CSF randomly covalently attached to a non-releasable, branched PEG reagent, PEG2-ru-40K-NHS:



PEG2-ru-40K-G-CSF

as well as to filgrastim (recombinant methionyl human G-CSF, Neupogen®) and rhG-CSF. See FIG. 8 for potency data for each of the representative compositions at 72 hours. The EC50 results for the same comparative *in-vitro* activity study are shown in FIG. 9. The activity data demonstrates that the potency (activity) of the exemplary releasable mono-conjugate PEG-GCSF compositions and the non-PEGylated G-CSF molecules, recombinant methionyl human GCSF and rhGCSF, are comparable over time, and further illustrates the enhanced activity of the releasable mono-conjugate compositions, C2-FMOC-PEG2-40K-GCSF-M and CG-FMOC-PEG-40K-GCSF-M, over the exemplary non-releasable branched mono-conjugate, PEG2-ru-40K-GCSF-M.

Example 5

In-Vivo Study

Neutrophil Recovery in Neutropenic Mice

[0241] The ability of test articles to stimulate the production of neutrophils was measured by administering the test article to neutropenic mice and counting the number of neutrophils at various time points to further confirm the potencies of the exemplary G-CSF compositions.

[0242] Mice (Charles River, CD1, females) were provided a standard diet and allowed to acclimatize for one week prior to initiation of the study. Animals were separately grouped based on the test article being administered, wherein animals were further grouped based on whether they received 100, 300 or 1000 µg/kg of test article.

[0243] Animals were made neutropenic by intraperitoneal administration of cyclophosphamide on days -4 and -1 (150 mg/kg and 100 mg/kg, respectively). At time 0 on day 1 (prior to dosing with test article) and at times 4, 12, 24, 36, 48, 60, 72, 96 and 144 hours, up to six animals from each group were sacrificed and the hematology panel run on the sample and the mean neutrophil values from sacrificed animals for each time point reported.

[0244] Tables 4A, 4B and 4C show the mean neutrophil counts of various doses of Neulasta® (pegfilgrastim), C2- FMOC-PEG2-40K-GCSF-A and CG- FMOC-PEG2-40K-GCSF-A, respectively. FIG. 10 provides the mean neutrophil counts at time points 48, 60, 72, 96 and

144 hours of Neulasta[®] (pegfilgrastim), CG- FMOC-PEG2-40K-GCSF-A, C2- FMOC-PEG2-40K-GCSF-A and vehicle, each at a dose of 1000 µg/kg.

Table 4A

Mean Neutrophil Counts of Various Doses of Neulasta[®] pegfilgrastim

Time (hr)	Mean Neutrophil Count (in 10 ³ /µL) of 100 µg/kg of Neulasta [®] pegfilgrastim	Mean Neutrophil Count (in 10 ³ /µL) of 300 µg/kg of Neulasta [®] pegfilgrastim	Mean Neutrophil Count (in 10 ³ /µL) of 1000 µg/kg of Neulasta [®] pegfilgrastim
0	0.143	0.19	0.087
4	0.233	0.228	0.268
12	0.404	0.136	0.052
24	0.183	0.162	0.243
36	0.118	0.532	1.082
48	2.063	1.624	2.205
60	6.825	16.56	NA
72	NA	18.253	15.25
96	13.007	8.95	9.56
144	1.67	1.258	3.42

Table 4B

Mean Neutrophil Counts of Various Doses of C2-FMOC-PEG2-40K-GCSF-A

Time (hr)	Mean Neutrophil Count (in 10 ³ /µL) of 100 µg/kg of C2-FMOC-PEG2-40K-GCSF-A	Mean Neutrophil Count (in 10 ³ /µL) of 300 µg/kg of C2-FMOC-PEG2-40K-GCSF-A	Mean Neutrophil Count (in 10 ³ /µL) of 1000 µg/kg of C2-FMOC-PEG2-40K-GCSF-A
0	0.13	0.146	0.116
4	0.152	0.215	0.172
12	0.068	0.232	0.098
24	0.182	0.336	0.113
36	1.698	0.212	0.388
48	0.695	1.192	0.35
60	6.635	10.6	10.825
72	4.748	7.793	12.963
96	5.486	4.245	11.098
144	2.798	3.233	1.883

Table 4C

Mean Neutrophil Counts of Various Doses of CG-FMOC-PEG2-40K-GCSF-A

Time (hr)	Mean Neutrophil Count (in $10^3/\mu\text{L}$) of 100 $\mu\text{g/kg}$ of CG-FMOC-PEG2-40K-GCSF-A	Mean Neutrophil Count (in $10^3/\mu\text{L}$) of 300 $\mu\text{g/kg}$ of CG-FMOC-PEG2-40K-GCSF-A	Mean Neutrophil Count (in $10^3/\mu\text{L}$) of 1000 $\mu\text{g/kg}$ of CG-FMOC-PEG2-40K-GCSF-A
0	0.096	0.103	0.13
4	0.128	0.14	0.255
12	0.157	0.126	0.12
24	0.207	0.192	0.472
36	0.318	0.288	0.197
48	0.467	1.358	1.102
60	4.03	7.01	10.1
72	4.58	12.402	18.36
96	5.143	7.398	17.5
144	1.453	3.553	2.924

[0245] As can be seen from the tables above and in FIG. 10 (1000 $\mu\text{g/kg}$ dose), both of the "A" conjugates, C2-FMOC-PEG2-40K-GCSF-A and CG-FMOC-PEG2-40K-GCSF-A, demonstrated mean neutrophil counts at varying dose amounts that were, on the whole, either superior to, or comparable with, Neulasta[®]. In reference to FIG. 10, at 60, 72 and 96 hours post-dosing, the CG-FMOC-PEG2-40K-GCSF-A showed a superior mean neutrophil response relative to Neulasta[®], as did C2-FMOC-PEG2-40K-GCSF-A at 60 and 96 hours post-dose, the indicating the advantageous nature of conjugates and conjugate mixtures such as provided herein.

Example 6

Releasable G-CSF Conjugates: Binding Kinetics Study

[0246] The binding kinetics of exemplary releasable G-CSF conjugates were examined and compared to native rh-G-CSF and Neupogen[®]. The compositions examined were as follows: Neulasta[®] (pegfilgrastim), rh-G-CSF, CG-FMOC-PEG2-40K-GCSF-A, CG-FMOC-PEG2-40K-GCSF-B, CG-FMOC-PEG2-40K-GCSF-C, CG-FMOC-PEG2-40K-GCSF-M, C2-FMOC-PEG2-40K-GCSF-A, C2-FMOC-PEG2-40K-GCSF-B, C2-FMOC-PEG2-40K-GCSF-C, and C2-FMOC-PEG2-40K-GCSF-M.

[0247] Experiments were carried out on a BioRad ProteOn™ system at 25°C. Goat anti-Human IgG was coupled to a GLM sensor chip using conventional amine coupling methods. In the first step, the surfaces were activated with sulfo-NHS/EDC for 5 minutes, followed by an injection of the Goat anti-human IgG in a buffer solution of 10 mM sodium acetate at pH 5.0 for 5 minutes, followed by a 5 minute injection of 1 molar ethanolamine as a blocking step. This coupling method resulted in the immobilization of ~7000 RU of mAb.

[0248] To test each G-CSF ligand sample, the G-CSF-Fc receptor construct was first captured onto the anti-human surface. 50 µg of G-CSF-Fc receptor was dissolved into 400 µL of the PBS running buffer including 0.01% Tween®-20 and 0.1 mg/ml Earle's Balanced Salt Solution (BSS). This stock of receptor (Apollo Cytokine Research Cat 1101I, lot 8G1X03T) was then diluted 100-fold and injected for 640 seconds over the anti-human IgG surface to capture the receptor onto the surface. Stable capture of ~700 RU of the G-CSF-Fc Receptor was confirmed.

[0249] The G-CSF samples were then tested for binding to the G-CSF-Fc receptor surface using a three-fold dilution series with 5 nM as the highest concentration at 25°C. Each G-CSF sample was freshly prepared immediately prior to the test (each sample was in the PBS buffer for only approximately 1 minute prior to testing). The G-CSF samples were injected at 50 µl/minute for 440 seconds, followed by a 1 hour dissociation phase. At the end of the dissociation phase, the surfaces were regenerated with 2X 12 second pulses of 1/200 dilution of phosphoric acid. The assay was then repeated by capturing a new aliquot of the G-CSF-Fc receptor to test the next sample.

[0250] The response data for each G-CSF sample were globally fit using a 1:1 interaction model. Given that the dissociation rate was very slow and the differences were indistinguishable for all of the G-CSF samples evaluated, the rate was fixed at $4.66 \times 10^{-6} \text{ s}^{-1}$ so that a better comparison could be made of the association rates (which were, in fact, different for some of the samples).

[0251] See Table 5 for binding association and equilibrium dissociation constants determined at 25°C. The values reported are the average of the three replicate surface studies and the standard deviation is the experimental standard deviation.

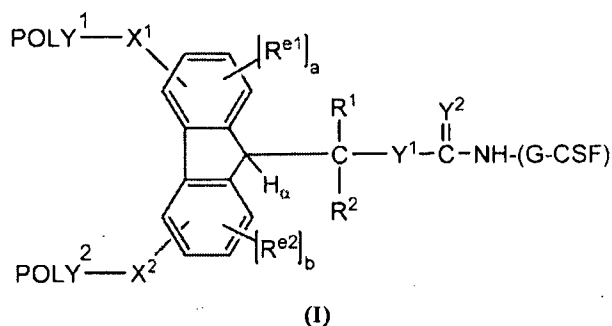
Table 5

Compound	K _a (M ⁻¹ s ⁻¹)	K _D (pM)
Native rh-G-CSF	(2.3 ± 0.1)E6	2.1 ± 0.1
Neupogen®	(2.1 ± 0.1)E6	2.3 ± 0.1
CG-FMOC-PEG2-40K-GCSF-A	(9.1 ± 0.6)E5	5.1 ± 0.3
CG-FMOC-PEG2-40K-GCSF-B	(5.4 ± 0.6)E5	8.7 ± 0.9
CG-FMOC-PEG2-40K-GCSF-C	(3.5 ± 0.4)E5	13.4 ± 1.4
C2-FMOC-PEG2-40K-GCSF-A	(6.6 ± 0.3)E5	7.1 ± 0.4
C2-FMOC-PEG2-40K-GCSF-B	(1.03 ± 0.01)E6	4.5 ± 0.1
C2-FMOC-PEG2-40K-GCSF-C	(7.8 ± 0.4)E5	6.0 ± 0.3
C2-FMOC-PEG2-40K-GCSF-M	(7.4 ± 0.3)E5	6.3 ± 0.3
CG-FMOC-PEG2-40K-GCSF-M	(7.3 ± 0.4)E5	6.4 ± 0.4

[0252] The data in the **Table 5** indicates that each of the exemplary releasable G-CSF mono-conjugate compositions evaluated possesses good binding to the G-CSF-Fc receptor. Moreover, as shown above, all of the releasable G-CSF mono-conjugate compositions were found to be extremely potent. The conjugates and conjugate compositions provided herein are well-defined; preferred conjugates are G-CSF mono-conjugates having a single, releasable, branched PEG moiety covalently attached to a single site (e.g., amino site) on the G-CSF molecule. Moreover, the *in-vivo* results in mice indicate enhanced potencies of exemplary releasable, branched mono-G-CSF conjugate conjugate compositions over the commercial product, pegfilgrastim.

IT IS CLAIMED:

1. A composition comprising a G-CSF moiety releasably attached to a branched water-soluble polymer at from one to three amino sites therein via a linkage selected from -O-C(O)-NH-, -O-C(S)-NH-, -S-C(O)-NH-, and -S-C(S)-NH-, wherein the -NH- indicates an amino group on the G-CSF moiety to which the branched water soluble polymer is releasably attached.
2. The composition of claim 1, wherein the composition substantially comprises the G-CSF moiety having the branched water-soluble polymer releasably attached at only two of its amino sites.
3. The composition of claim 1, wherein the composition substantially comprises the G-CSF moiety having the branched water-soluble polymer releasably attached at only a single amino site of the G-CSF moiety.
4. The composition of claim 1, wherein the branched water-soluble polymer has a weight average molecular weight in a range of about 5,000 Daltons to about 100,000 Daltons.
5. The composition of any one of claims 1-4, wherein the G-CSF moiety releasably attached to a branched water-soluble polymer corresponds to the structure:



wherein:

- POLY¹ is a first water-soluble polymer;
- POLY² is a second water-soluble polymer;
- X¹ is a first spacer moiety;
- X² is a second spacer moiety;

H_α is an ionizable hydrogen atom;

R¹ is H, SO₃H, or an organic radical;

R² is H, SO₃H, or an organic radical;

(a) is either zero or one;

(b) is either zero or one;

R^{e1}, when present, is a first electron altering group;

R^{e2}, when present, is a second electron altering group; and

Y¹ is O or S;

Y² is O or S; and

(G-CSF) is a residue of an amine-containing G-CSF moiety (where the NH-G-CSF indicates the amine-residue thereof).

6. The composition of claim 5, wherein the X¹ and X² spacer moieties are independently selected from -NHC(O)-(CH₂)-, -NHC(O)-(CH₂)₂-, -NHC(O)-(CH₂)₃-, -NHC(O)-(CH₂)₄-, -NHC(O)-(CH₂)₅-, -NHC(O)-(CH₂)₆-, -NHC(O)(CH₂)C(O)NH(CH₂)₂-, -NHC(O)(CH₂)₂C(O)NH(CH₂)₂-, -NHC(O)(CH₂)₃C(O)NH(CH₂)₂-, -NHC(O)(CH₂)₄C(O)NH(CH₂)₂-, -NHC(O)(CH₂)₅C(O)NH(CH₂)₂-, -NHC(O)(CH₂)₆C(O)NH(CH₂)₂-, -C(O)-O-(CH₂)-, -C(O)-O-(CH₂)₂-, -C(O)-O-(CH₂)₃-, -C(O)-O-(CH₂)₄-, -C(O)-O-(CH₂)₅-, -C(O)-O-(CH₂)₆-, -(CH₂)C(O)NH(CH₂)₂-, -(CH₂)₂C(O)NH(CH₂)₂-, -(CH₂)₃C(O)NH(CH₂)₂-, -(CH₂)₄C(O)NH(CH₂)₂-, -(CH₂)₅C(O)NH(CH₂)₂-, -(CH₂)₆C(O)NH(CH₂)₂-, and -C(O)NH-(CH₂)₂-.

7. The composition of claim 6, wherein the X¹ and X² spacer moieties are the same.

8. The composition of claim 7, wherein the X¹ and X² spacer moieties are both and -C(O)NH-(CH₂)₂-.

9. The composition of claim 6, wherein X¹ is -C(O)NH-(CH₂)₂- and X² is NHC(O)(CH₂)₃C(O)NH(CH₂)₂-.

10. The composition of claim 5, wherein R¹ and R² are both H.

11. The composition of claim 5, wherein Y¹ and Y² are both O.

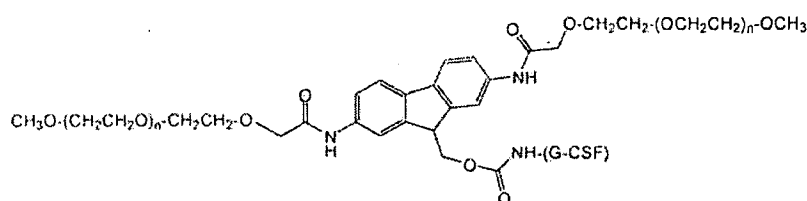
12. The composition of claim 5, wherein R^{e1} , when present, is an electron-withdrawing group.

13. The composition of claim 5, wherein R^{e2} , when present, is an electron-withdrawing group.

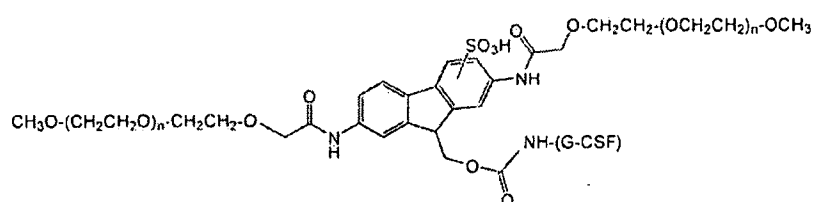
14. The composition of claim 12 or claim 13, where R^{e1} and R^{e2} , if present, either singly or collectively, is independently selected from a halogen, $-C(O)H$, $-C(O)R$, $-C(O)OR$, $-C(O)OH$, $-C(O)X$, where X is a halo group, $-CF_3$, $-C\equiv N$, and $-SO_3H$, where R is an organic radical.

15. The composition of claim 14, wherein R^{e1} is $-SO_3H$.

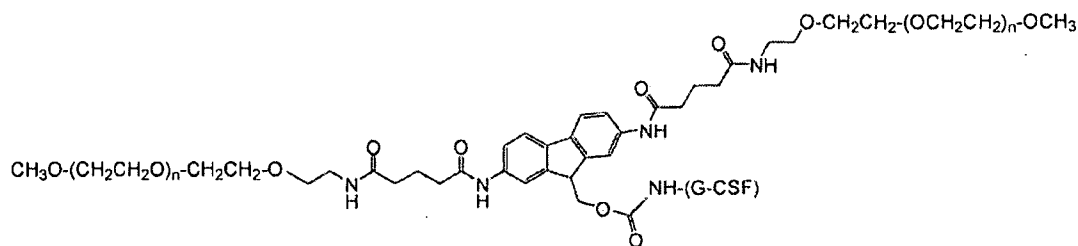
16. The composition of any one of claims 1-4, wherein the G-CSF moiety releasably attached to a branched water-soluble polymer corresponds to a structure selected from:



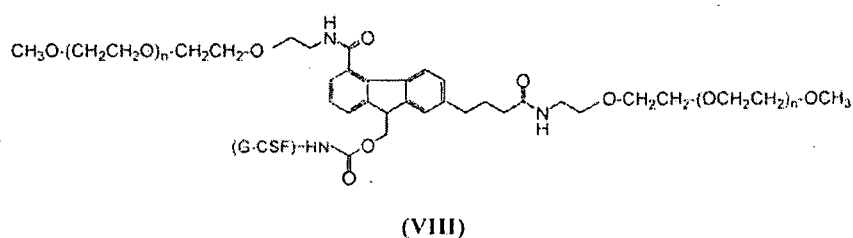
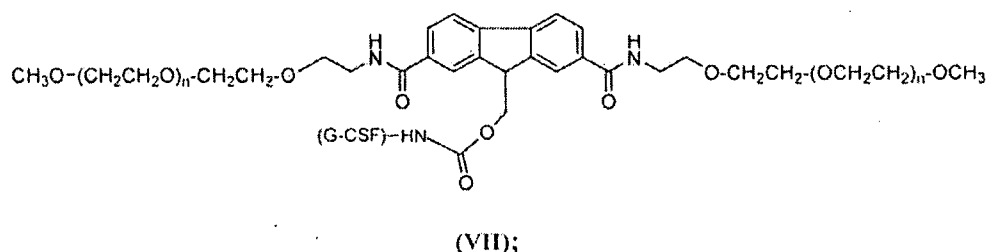
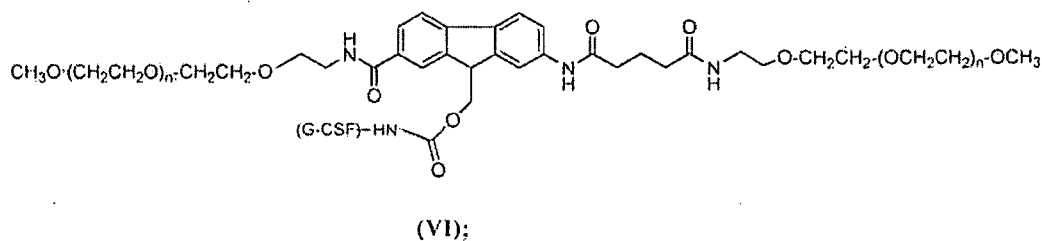
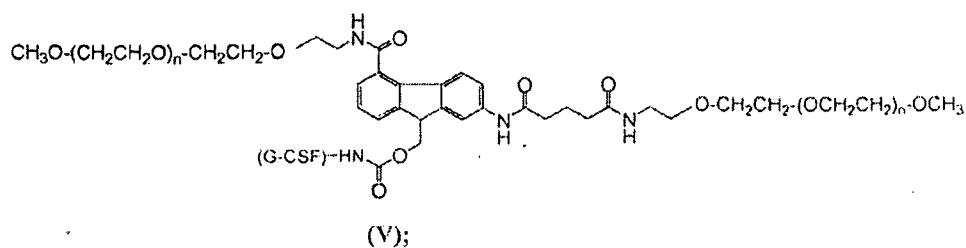
(II)



(III)

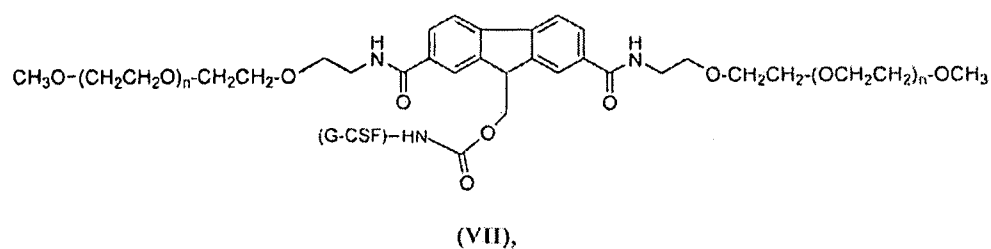


(IV);

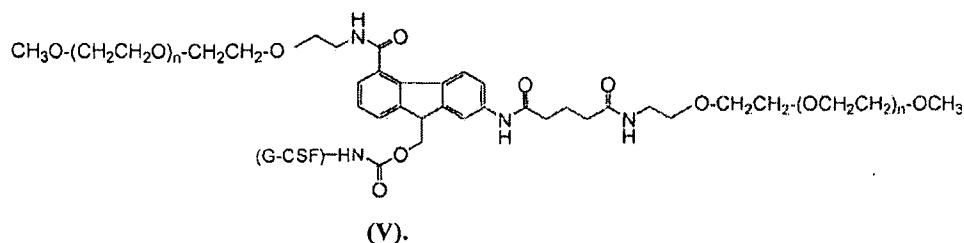


wherein, for each structure and in each instance, (n) is independently an integer from 4 to 1500, and (G-CSF) is a residue of a G-CSF moiety.

17. The composition of claim 16, wherein the G-CSF moiety releasably attached to a branched water-soluble polymer corresponds to a structure selected from:



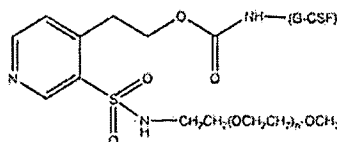
and



18. The composition of claim 17, wherein the G-CSF moiety is selected from native human G-CSF and recombinant G-CSF.

19. The composition of claim 18, wherein the G-CSF moiety is full length recombinant G-CSF that is releasably attached to the branched water-soluble polymer at a lysine group selected from K17, K24, K35, K41 and the N-terminus.

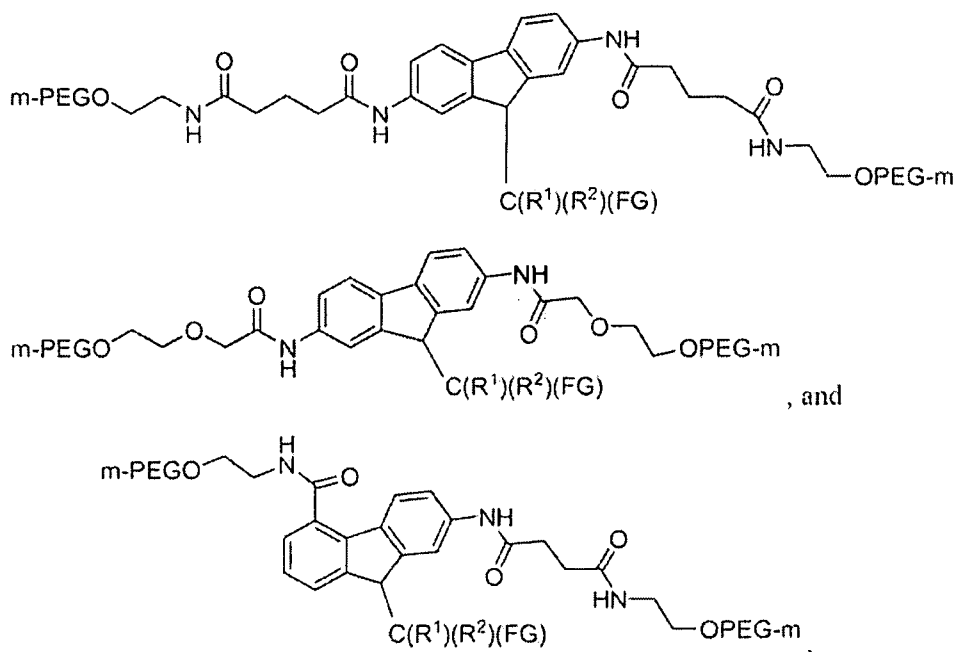
20. The composition of any one of claims 1-4, wherein the G-CSF moiety releasably attached to a branched water-soluble polymer corresponds to the structure:



wherein, in each instance, (n) is independently an integer from 4 to 1500.

21. A method of making a composition of any one of claims 1-20, comprising:
 contacting (i) an amine-reactive, branched water-soluble polymeric reagent suitable for forming a releasable covalent bond to an amino group, with (ii) a G-CSF moiety comprising at least one amino group, under reaction conditions suitable for forming a releasable covalent bond selected from -O-C(O)-NH-, -O-C(S)-NH-, -S-C(O)-NH-, and -S-C(S)-NH-, wherein the -NH- indicates the amino group on the G-CSF moiety to which the branched, water soluble polymer is releasably attached.

22. The method of claim 21, wherein the polymeric reagent has a structure selected from the group consisting of:



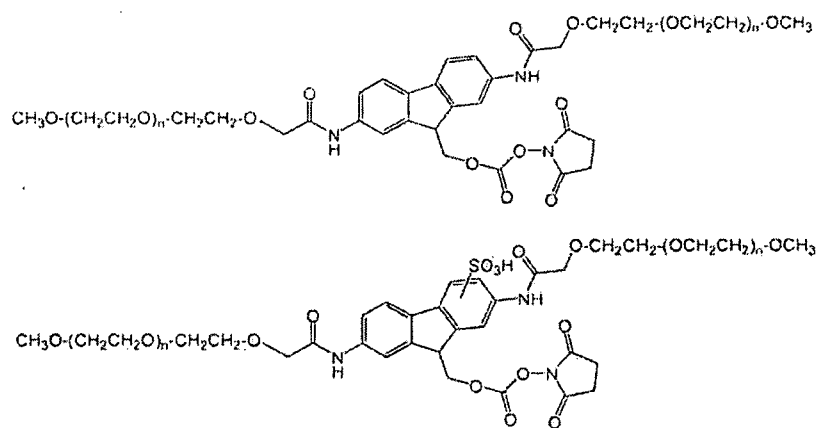
wherein, in each instance:

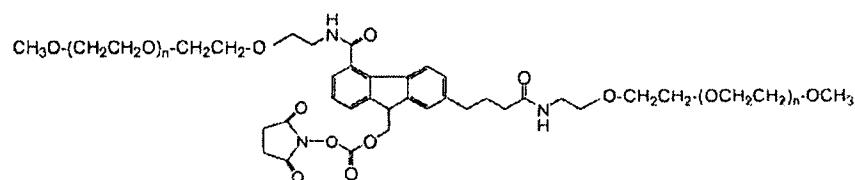
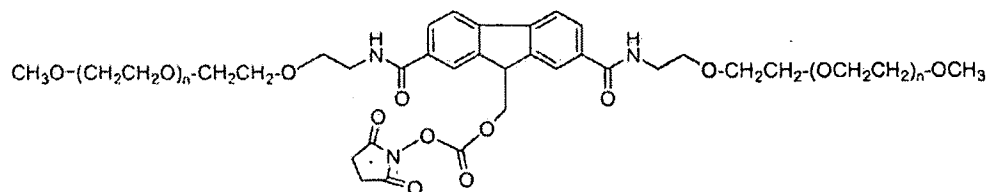
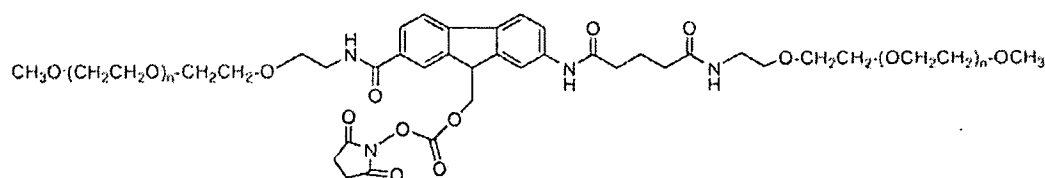
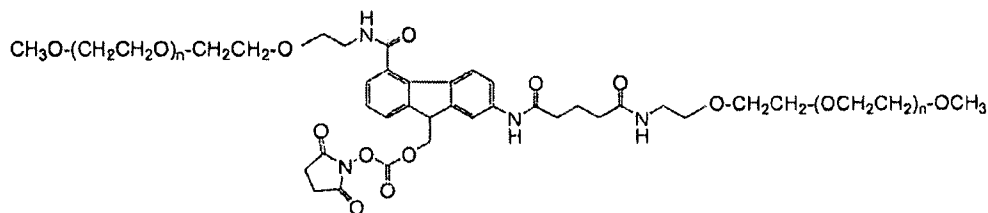
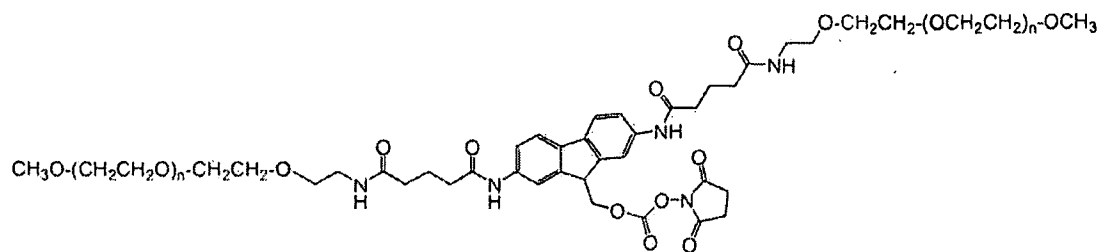
(FG) is a functional group capable of reacting with an amino group of a G-CSF moiety to form a releasable linkage selected from from -O-C(O)-NH-, -O-C(S)-NH-, -S-C(O)-NH-, and -S-C(S)-NH-,

R^1 is H, SO_3H , or an organic radical; and

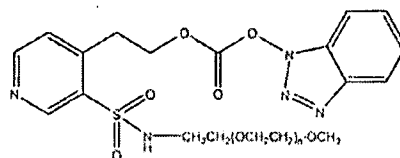
R^2 is H, SO_3H , or an organic radical.

23. The method of claim 21, wherein the polymeric reagent is selected from:





; and



wherein, for each structure and in each instance, (n) is independently an integer from 4 to 1500.

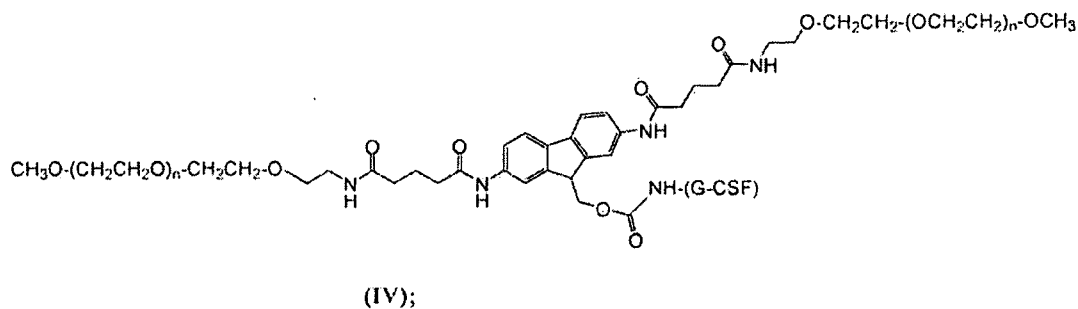
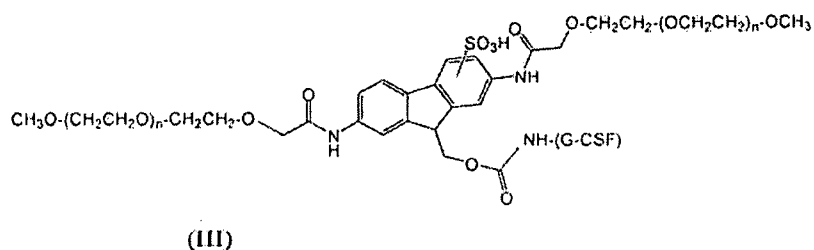
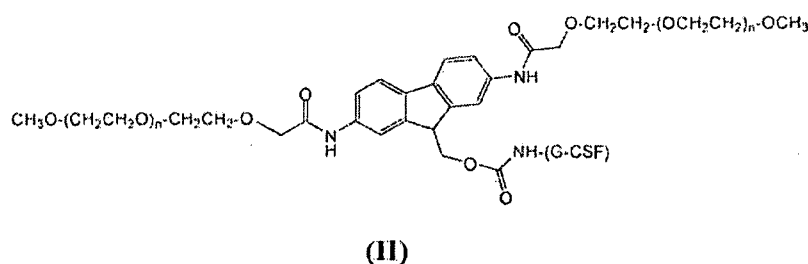
24. The method of any one of claims 21-23, wherein the polymeric reagent is contacted with the G-CSF moiety at an equimolar amount based upon moles of amine-reactive groups in the polymeric reagent versus moles of reactive amines in the G-CSF moiety.

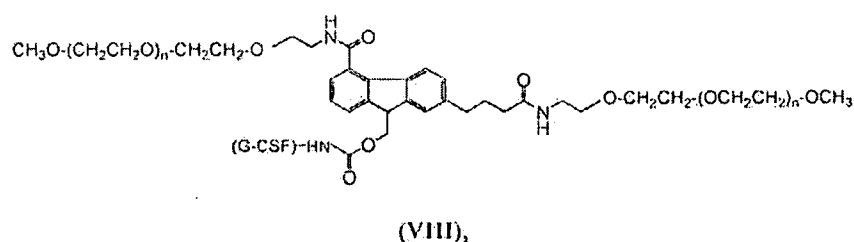
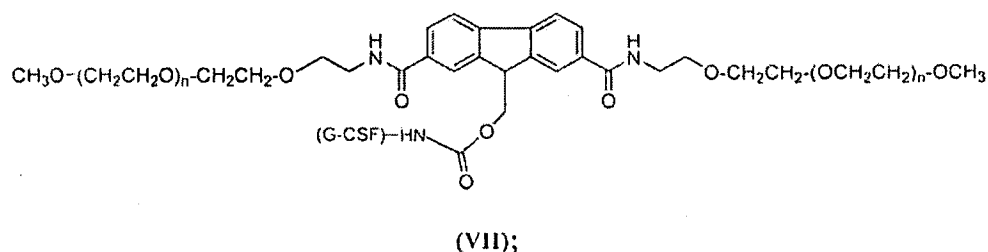
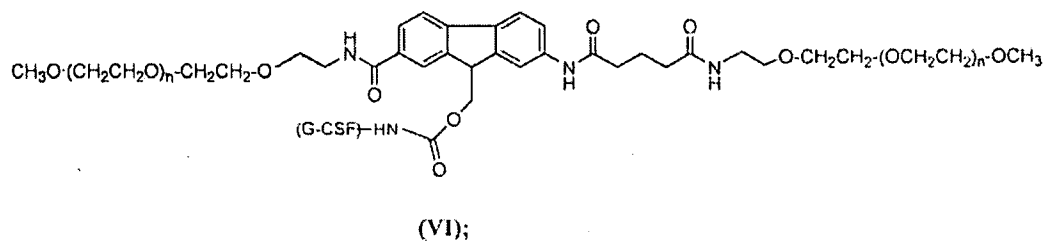
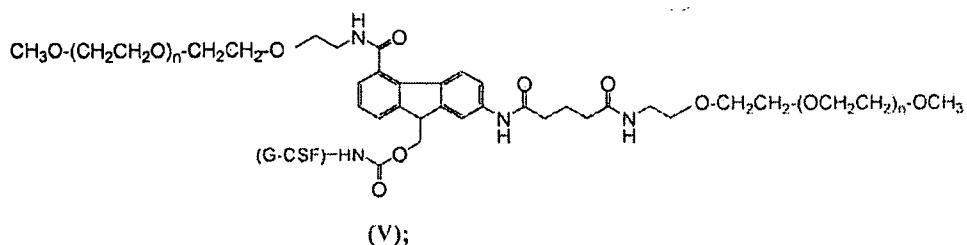
25. The method of any one of claims 21-23, wherein the polymeric reagent is contacted with the G-CSF moiety at a molar ratio (based upon reactive groups) of about 1:1 to 10:1.

26. The method of any one of claims 21-25, wherein the contacting is carried out in an aqueous buffer at a pH from about 7 to about 9.

27. A pharmaceutical preparation comprising the composition of any one of claims 1-20 and a pharmaceutically acceptable excipient.

28. A pharmaceutical preparation of claim 27, comprising a majority of a single positional isomer of a G-CSF moiety releasably attached at a single of its amino sites to a branched water-soluble polymer and having a structure selected from

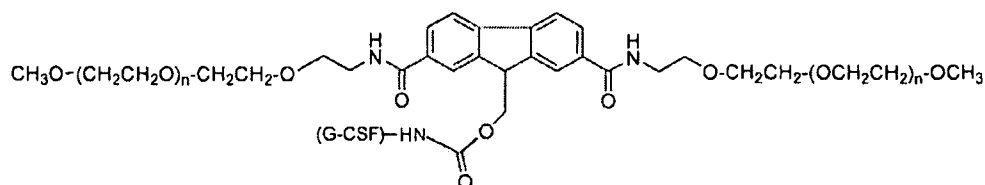




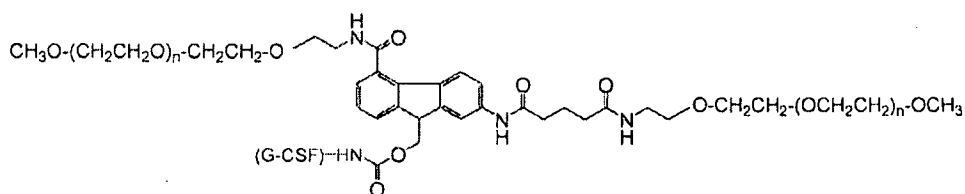
where the composition may possess additional positional isomers having the branched water soluble polymer releasably attached to a different single attachment site on the G-CSF moiety, and for each structure and in each instance, (n) is independently an integer from 4 to 1500.

29. The pharmaceutical preparation of claim 28, substantially comprising the single positional isomer, where the composition is substantially free of additional positional isomers having the branched water soluble polymer releasably attached to a different single attachment site on the G-CSF moiety.

30. The pharmaceutical preparation of claim 28 or 29, wherein the single positional isomer of a G-CSF moiety releasably attached at a single of its amino sites to a branched water-soluble polymer has a structure corresponding to:



or



31. A composition comprising a mixture of releasable mono-G-CSF conjugates comprising a G-CSF moiety releasably attached to a branched water-soluble polyethylene glycol (PEG) polymer at a single of its amino sites via a linkage selected from -O-C(O)-NH-, -O-C(S)-NH-, -S-C(O)-NH-, and -S-C(S)-NH-, wherein the NH- indicates an amino group on the G-CSF moiety to which the branched water soluble polymer is releasably attached, wherein each of the individual mono-G-CSF conjugates comprised in the mixture possesses a different release rate of G-CSF *in-vitro* under physiological conditions.

32. A composition of any one of claims 27-31 for use in administration to a human or animal subject.

33. The composition of claim 32 for use in administration to a human subject for treatment of neutropenia.

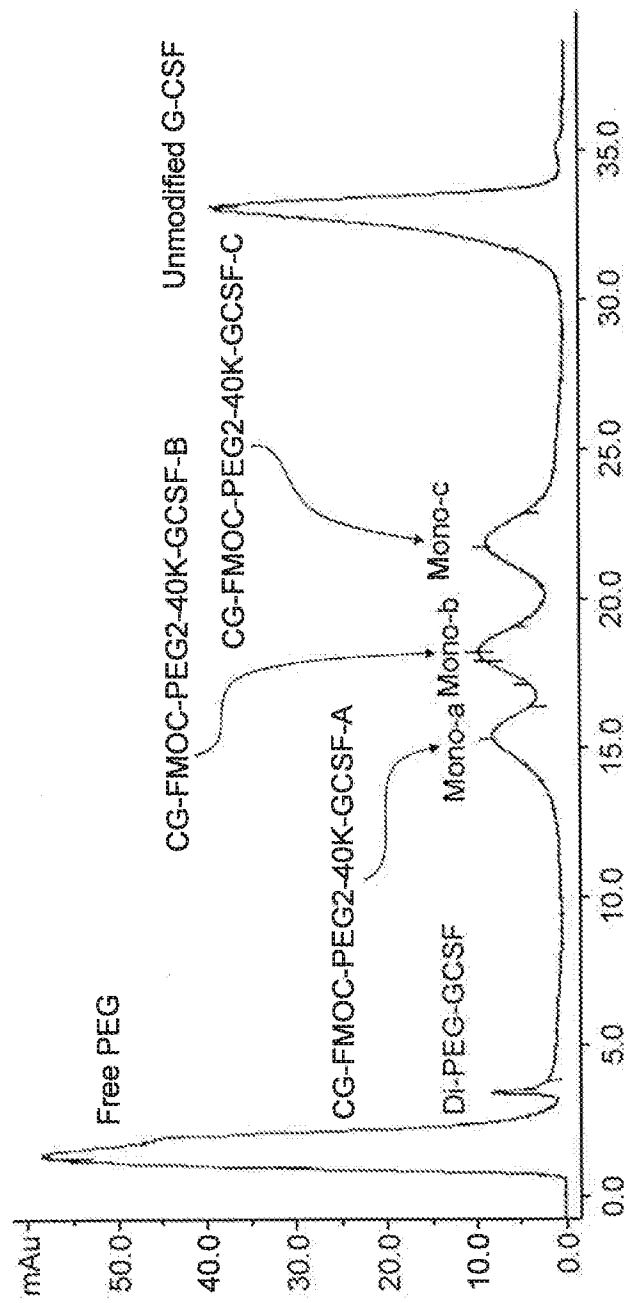


FIG. 1

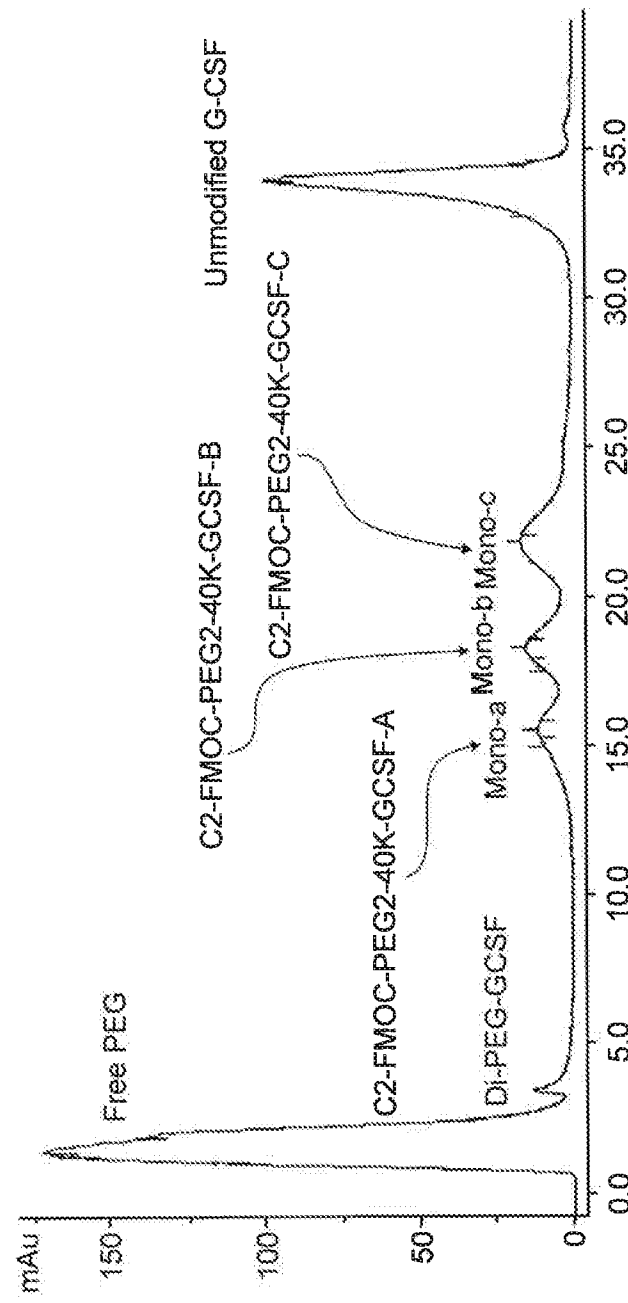


FIG. 2

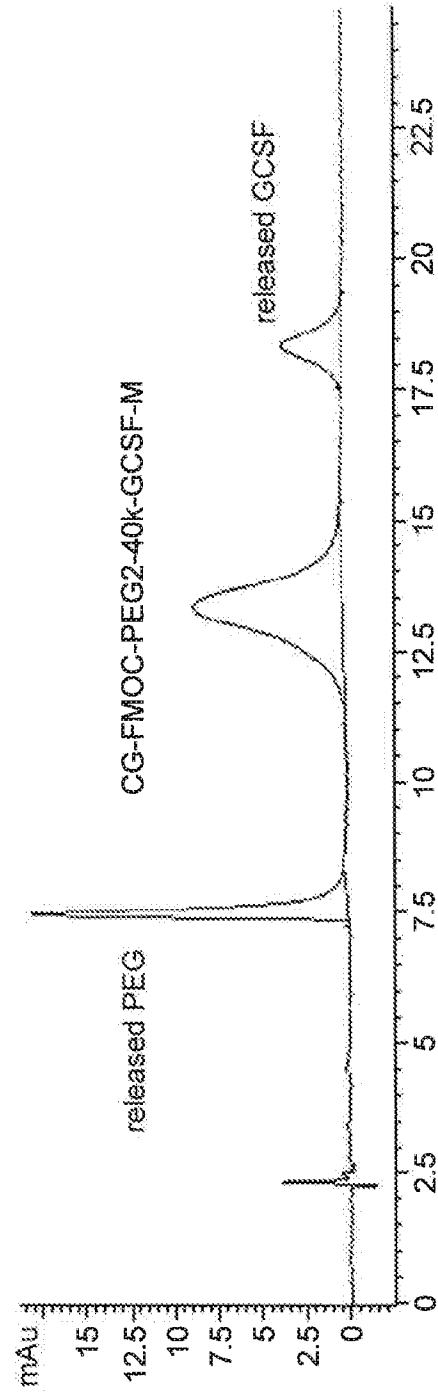


FIG. 3

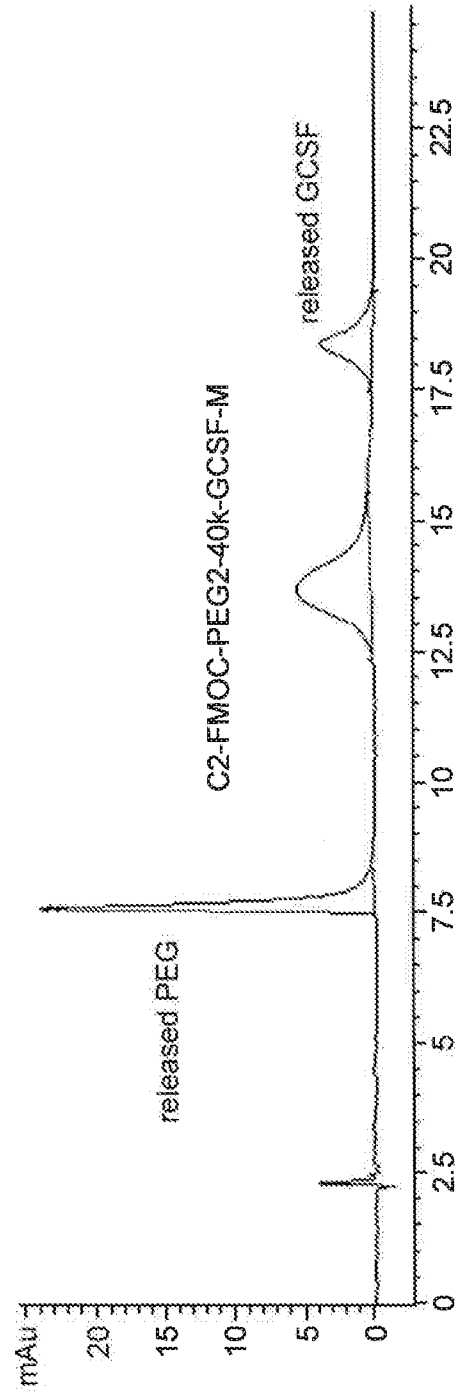


FIG. 4

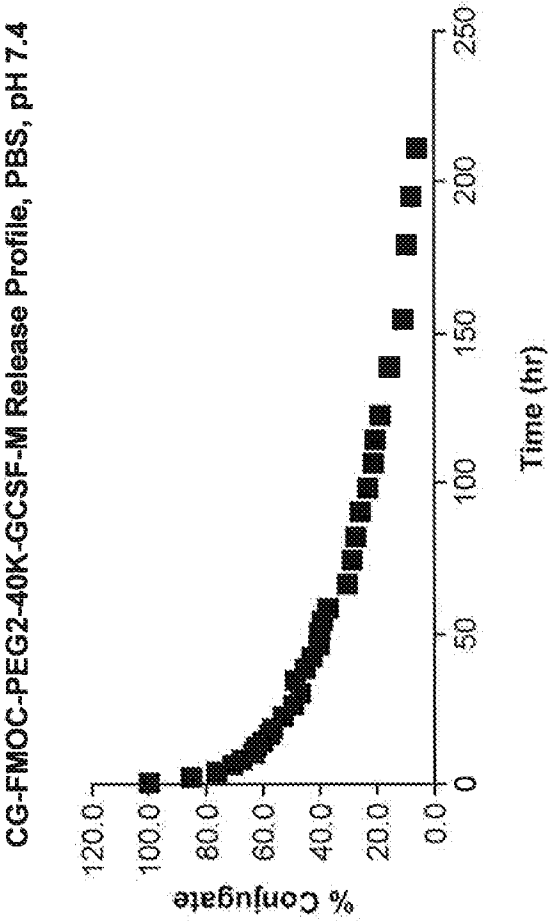


FIG. 5A

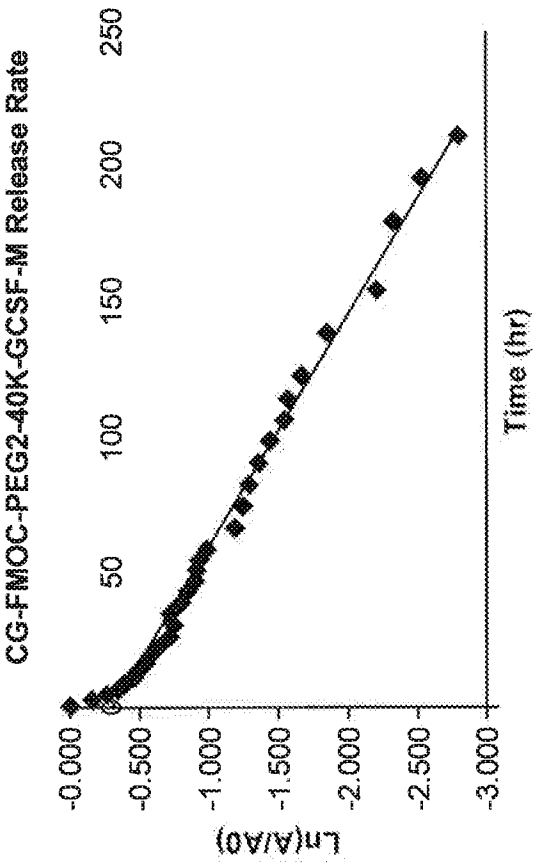


FIG. 5B

C2-FMOC-PEG2-40K-GCSF-M Release Profile, PBS, pH 7.4

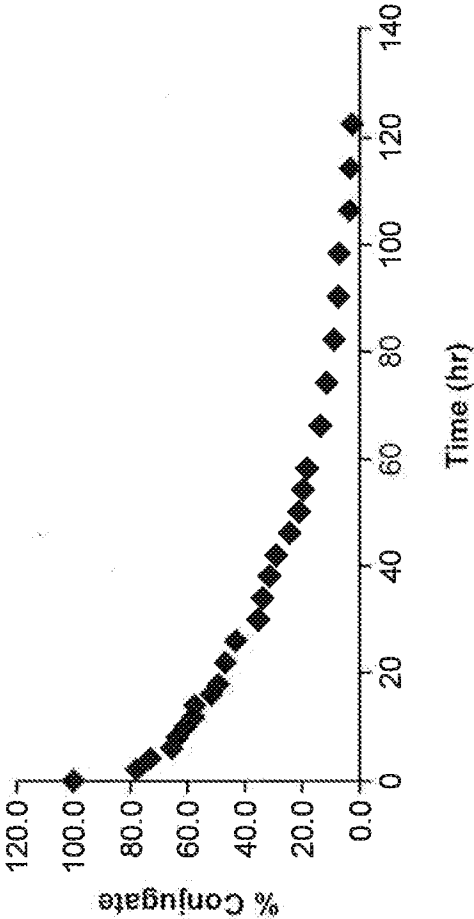


FIG. 6A

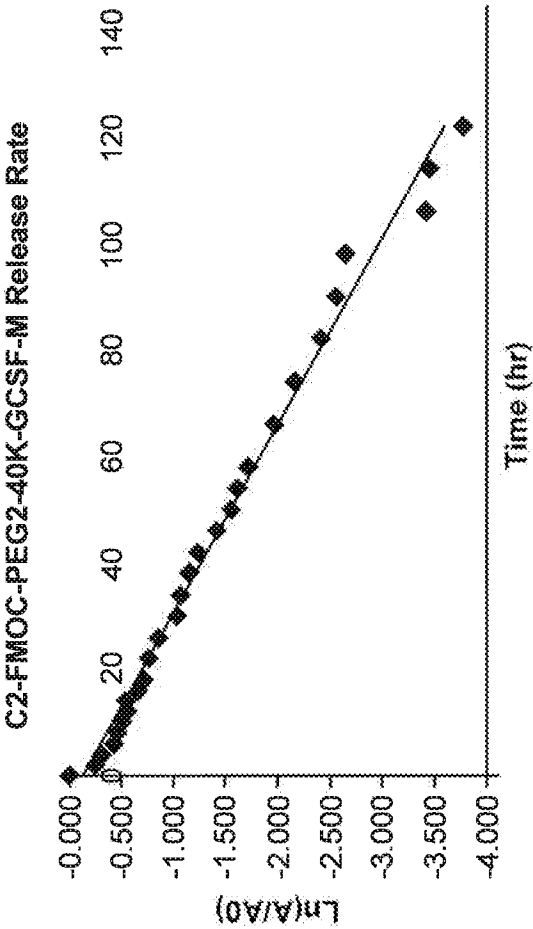


FIG. 6B

Proliferation of M-NFS-60 cells at 72 h
in response to releasable PEG-G-CSF

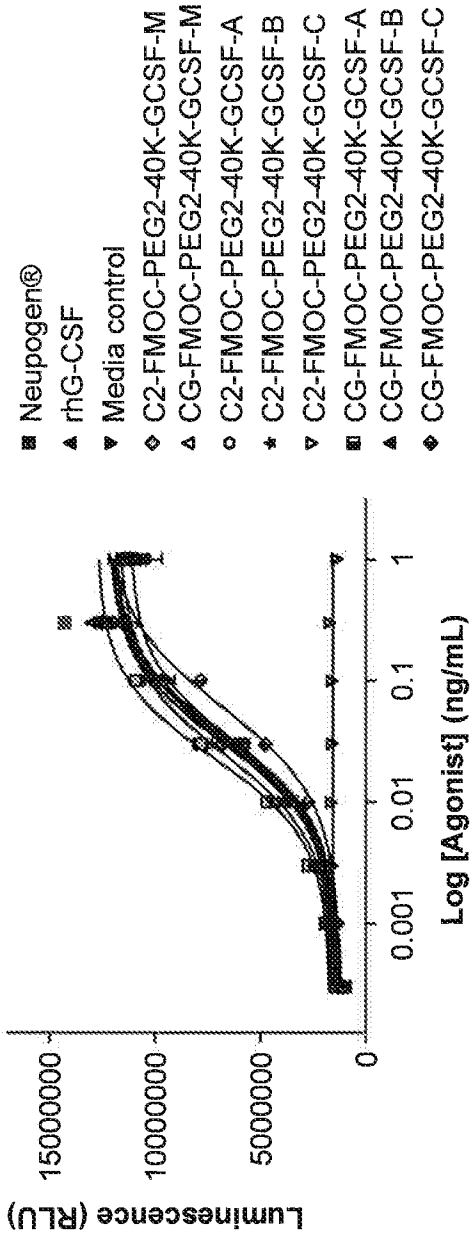


FIG. 7

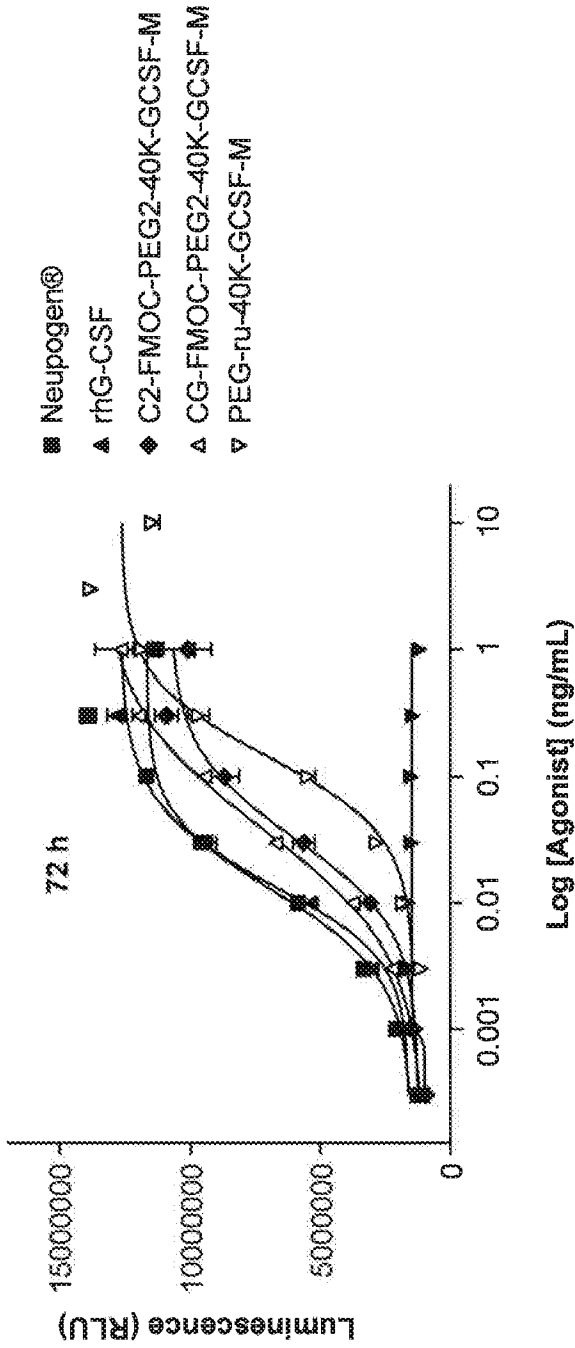


FIG. 8

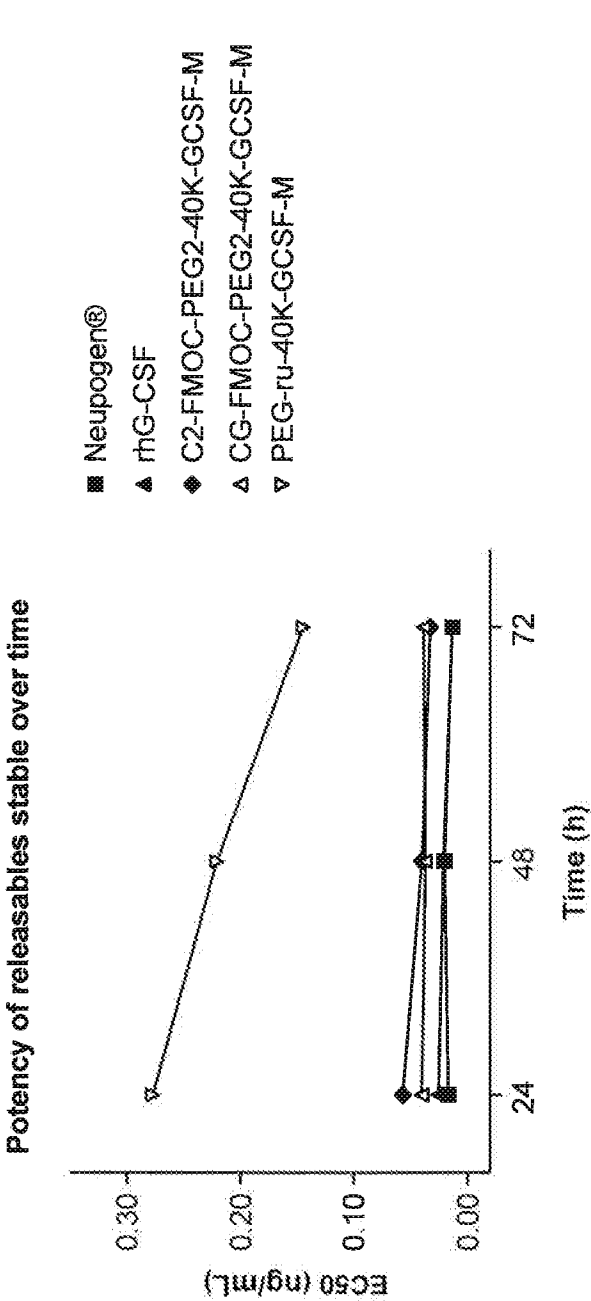


FIG. 9

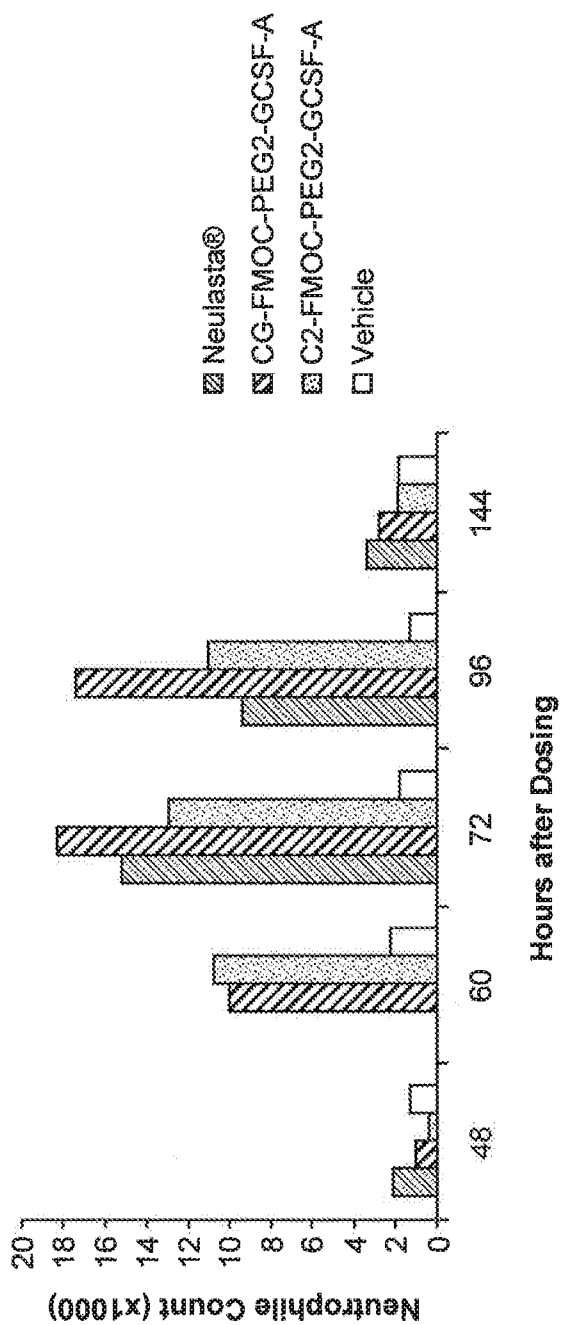


FIG. 10