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(54) **Title:** NANOFORMULATION OF G-CSF FOR NON-PARENTERAL DELIVERY

(57) **Abstract:** The present invention relates to novel protein nanoparticle compositions for oral administration with improved intesti-  
nal absorption of the protein.



**Abstract**

The present invention relates to novel protein nanoparticle compositions for oral administration  
5 with improved intestinal absorption of the protein.

**Background**

Biopharmaceuticals are an important class of drugs for the treatment of numerous diseases due  
to their ability to provide selective and potent response in the body. For a number of diseases,  
10 biopharmaceuticals are the only medicines that can cure or ameliorate the disease.

Granulocyte colony-stimulating factor (G-CSF) is a cytokine. In the human body, G-CSF is  
mainly produced by macrophages and monocytes. G-CSF controls differentiation of precursor  
cells to neutrophils, their proliferation, and stimulates the release of mature neutrophils from the  
15 bone marrow. Native human G-CSF is a glycoprotein with a short glycane chain attached to  
Thr133 (O-glycosylation). It has a typical cytokine structure: a four helix-bundle, which is  
stabilized by two disulphide bridges.

Chemotherapeutics, with the exception of target specific monoclonal antibodies, act non-  
20 selectively upon cells with a high replication rate causing neutropenia as an adverse side effect.  
The resulting drop in neutrophil count increases the risk of severe life-threatening infections, and  
is the major dose-limiting factor of systemic chemotherapy. For the past two decades,  
chemotherapy-induced neutropenia was successfully prevented by the use of granulocyte  
colony-stimulating factor (G-CSF), which increases the neutrophil count and thereby prevents  
25 severe infections.

Four G-CSF variants have been approved for medical use so far: filgrastim, lenograstim,  
nartograstim and pegfilgrastim. Filgrastim and lenograstim, both recombinant human G-CSFs,  
are first generation pharmaceuticals with equivalent efficacy. The improved second generation  
30 therapeutic forms, nartograstim (KW-2228) and pegfilgrastim, followed. In nartograstim, the  
amino acid sequence manipulation resulted in increased granulopoietic activity. Protein  
modification of filgrastim by pegylation increased the hydrodynamic radius to the degree where  
renal clearance is substantially reduced.

35 All above mentioned G-CSFs are administered parenterally, so that there are currently no oral  
formulations of G-CSF available. Major constraints in this regard are the poor absorption as well  
as the rapid and extensive degradation of G-CSF in the gastrointestinal tract. The poor  
absorption is mostly due to G-CSF's large molecular size and low lipophilicity, which limits its  
permeability across the intestinal epithelium.

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The parenteral administration, as practiced with the current G-CSF dosage forms on the market, is also inconvenient for patients. As this route of administration may be accompanied with numerous complications such as local irritation at the injection site, high fluctuations in plasma concentrations, and higher frequency of administration, the patient compliance is often poor.

- 5 Apart from the inconvenience for the patients, parenteral delivery systems are also more expensive in terms of production and drug administration through health care professionals. Many research studies are therefore directed towards the development of other pharmaceutical forms for proteins, which could be delivered non-invasively, either through the lungs (pulmonary), nose (nasal), skin (transdermal) or mouth (oral).

10

From the standpoint of patient compliance, the peroral route would be the most convenient. Oral delivery also offers more possibilities for achieving the desired pharmacokinetic profiles of the drug in the body. In addition, several studies have reported that orally delivered protein could more closely resemble the physiological action of endogenous proteins. Examples are proteins

15 that target the liver or enter the systemic circulation via the portal vein (e.g. "gut hormones" such as incretine).

20

Extensive research is performed worldwide on the development of pharmaceutical formulations for oral delivery of biopharmaceuticals with enhanced bioavailability. One of the most commonly used methods for the delivery of therapeutic proteins, G-CSF included, is the conjugation of the protein with PEG, i.e., the so-called PEGylation, lipidisation, or attaching peptides that cross the cell membrane (cell penetrating peptides, CPP), or ligands that recognize specific receptors and transporters on intestinal epithelial cells. However, protein modifications such as PEGylation may induce changes in the physico-chemical properties of the protein, e.g., they may lead to an

25 increase in size and molecular weight of the protein molecule, changes in conformation, steric hindrance of intermolecular interactions, increased hydrophilicity, and changes in electrostatic binding properties, all of which may affect the pharmacological behavior of these conjugates. A particular disadvantage is the prolonged circulation time of PEGylated proteins due to a decreased rate of clearance by the kidney, and/or a reduction of proteolysis and opsonisation.

30 Moreover, PEGylation often results in a loss of activity and binding affinity with the intended drug-target, and may further aggregation. All of these disadvantages make the use of PEGylated proteins unattractive and decrease its usability.

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Another known approach involves the use of specific low molecular weight compounds, so-called delivery agents, which are derivatives of amino acids and di-peptides. They interact weakly and non-covalently with proteins that partly and reversibly change the conformation of the protein. Such partly folded state of protein exhibits greater flexibility and lipophilicity, which are known key factors for increasing protein permeability across cell membranes; however, the potential problem of this approach is the requirement of large amounts of delivery agent per

40 dose, which raises questions about the safety of their use.

The use of absorption enhancers to increase the absorption of active ingredients through biological barriers is a further known approach. The most common absorption enhancers are bile acid salts (sodium deoxycholate, sodium taurocholate, sodium glycocholate), medium-chain fatty acids (oleic, linoleic, caprylic and caproic acid and mono and di-glycerides), non-ionic and ionic surfactants (polyoxyethylene ethers, sodium lauryl sulphate), salicylates, chelating agents (EDTA, alginates), nitric oxides, some polymers (chitosan, thiolated chitosan) and others. They involve several mechanisms of action, including changes in membrane fluidity, decrease in mucus viscosity, the leakage of protein through membranes, opening of tight junctions or otherwise disturbing the integrity of the mucosal membrane. This strategy is thus limited by the fact that the absorption enhancers may cause membrane damage, interfere with normal physiological processes in the gastrointestinal tract and facilitate the absorption of other, potentially undesirable substances.

Reduced extent of protein absorption from gastrointestinal tract may be also a consequence of protein degradation. The problem is, however, less pretentious and can be addressed to some extent by co-administering enzyme inhibitors or by using a variety of encapsulation and coating techniques. The most commonly used enzyme inhibitors are those based on amino acids (N-acetylcystein, boro-leucine, boro-valine, boro-alanine), peptides (bacitracin, bestatin, leupeptin, antipain, pepstatin, chymostatin, amastatin) and polypeptides (aprotinin, soybean trypsin inhibitor, chicken ovomucoid, casein). Complexing agents (EDTA) and some mucoadhesive polymers also display enzyme inhibitory activity. However, a problem of this approach is the disturbance of digestion of nutritive proteins, and stimulation of protease secretion as a result of feedback mechanism etc.

The incorporation of proteins into the carrier systems represents another approach to increase the absorption of protein from the gastrointestinal tract. Liposomes are one of the first carrier systems developed for targeted delivery of active ingredients, but their use as carriers for protein drugs has been less extensive. The main disadvantages of liposomal delivery of proteins include limited stability of liposomes and degradation of proteins during formulation since organic solvents are required for liposome production.

At the moment, nanoparticle (NP) systems are investigated for protein delivery. They consist of a carrier material that forms a "network" in which the protein is incorporated and thus partly protected from the aggressive environment in the gut. Polymeric NPs have been widely investigated as carriers for drug delivery. Much attention has been given to NPs made of synthetic biodegradable polymers such as polycaprolactone, polylactide and polyglycolide due to their biocompatibility. However, these NPs are not ideal carriers for hydrophilic drugs such as peptides and proteins because of their hydrophobic nature, which makes it difficult to prepare a formulation. In particular, the emulsion methods typically demand the use of organic solvents,

which can irreversibly change the activity of the therapeutic protein. From this standpoint, hydrophilic polymers of natural origin such as chitosan, alginate, dextran and the like are more suitable. These are polyionic polymers, polyelectrolytes, which have the ability to associate with the oppositely charged polyelectrolytes to form a gel under certain conditions, thus resulting in the formation of nanoparticles/nanocomplexes. Reported are nanoparticle formation by complex coacervation, polyelectrolyte or polyionic complexation, ionotropic gelation and others.

The incorporation of proteins into nanoscale delivery systems such as nanoparticles (NPs) remains a challenge, and several key requirements need to be observed during the development in order to obtain a suitable nano-sized delivery system. Above all, it is important to preserve the biological activity of the protein, which generally necessitates the use of aqueous media and applying only mild conditions during the production process; the NPs should preferably have a size of less than 500 nm, and should be colloidally stable; the protein association efficiency of the NPs should be high in order to obtain a NP formulation with a high final protein load per NP mass. These key requirements can only be achieved by an optimal chemical composition and architecture of the NPs. The selection and characteristics of the polymer employed further determines the choice of NP production and their final characteristics.

Almost all studies are focused on the delivery of peptides (calcitonin, parathyroid hormone (PTH), glucagon-like peptide (GLP-1) and GLP-1 analogs) and small proteins such as insulin. However, in comparison to G-CSF with a size around 18.8 kDa, insulin is a relatively small protein of a size of 6 kDa. As the different proteins (particular proteins of different size) may also differ considerably in their physico-chemical properties, the formulation of peptides or insulin into NPs cannot simply be applied to other proteins such as G-CSF. Therefore, an individual approach for formulating nanoparticulate delivery systems is required for any given bioactive protein.

Consequently, there is an ongoing need for an effective peroral delivery system for proteins, which avoids the known disadvantages associated with parenteral protein formulations such as the G-CSF formulations described above, and which enhances patient compliance. The present invention overcomes the aforesaid problems by providing a pharmaceutical composition of a protein such as G-CSF or erythropoietin, suitable for oral administration.

### Summary of the invention

The present invention relates to novel nanoparticle compositions with improved intestinal absorption of the proteins such as the protein granulocyte colony-stimulating factor (G-CSF), or erythropoietin. The composition contains a protein, e.g., G-CSF or erythropoietin, and an absorption enhancer, which are entrapped between two polyelectrolyte polymers having

opposite charges, such as chondroitin sulphate as negatively charged and trimethylchitosan as positively charged polymer.

By protecting the protein drug in carrier material, increasing the passage through mucosal surfaces and promoting the intestinal epithelial absorption, the formulation enhances the pharmacological availability of the poorly absorbable protein drug (G-CSF, or erythropoietin) when administered intraduodenally. This improvement is possible by using a combined approach of specific polymers, absorption enhancer and nanoformulation systems which act synergistically.

### Figure Legends

Figure 1: The absolute neutrophil count (ANC) after intraduodenal application of G-CSF-loaded nanoparticles of the present invention and placebo in rats. Asterisk indicates statistically significant difference.

Figure 2: The absolute neutrophil count (ANC) after intraduodenal application of free G-CSF in solution and placebo in rats.

Figure 3: The absolute neutrophil count (ANC) after subcutaneously administered G-CSF solution in rat at doses 1 µg/rat and 10 µg/rat.

### Detailed description of the invention

The present invention provides a composition containing a protein, e.g., G-CSF, or erythropoietin, and at least one absorption enhancer, e.g., cycloalkyl maltoside (CYMAL), which are entrapped between two polyelectrolyte polymers having opposite charges, for example chondroitin sulphate as negatively charged and trimethylchitosan as positively charged polymer.

#### G-CSF

The term "G-CSF" as used herein refers to Granulocyte-Colony Stimulating Factor and may be a natural or recombinant G-CSF, recombinant human G-CSF (rhG-CSF), or any protein or peptide having *in vivo* biological activity of the G-CSF glycoprotein, e.g., a wild type or mutant G-CSF, a G-CSF peptidomimetic, or a G-CSF fragment. The species from which G-CSF is derived can be animal, mammal or human species. Human G-CSF is, however, preferred. Human G-CSF comprises the known human Granulocyte-Colony Stimulating Factor having 174 amino acids. It further comprises human G-CSF in its naturally glycosylated state, i.e., including all carbohydrate side chains, in particular glycosylation at Thr133. The abbreviation "G-CSF" as used herein stands for G-CSF as defined above.

EPO

The term "EPO" as used herein refers to erythropoietin and may be a natural or recombinant EPO, recombinant human EPO (rhEPO), or any protein or peptide having *in vivo* biological activity of the EPO glycoprotein, e.g., a wild type or mutant EPO, an EPO peptidomimetic, an EPO fragment, or an EPO conjugate as provided herein. The species from which EPO is derived can be animal, mammal or human species. Human EPO is, however, preferred. Human EPO comprises the known human erythropoietin having 165 amino acids after posttranslational cleavage of the N-terminal signal peptide of 27 amino acids and the C-terminal arginine. It further comprises human EPO in its naturally glycosylated state, i.e., including all carbohydrate side chains.

Absorption Enhancers

Preferred absorption enhancers of the present invention are cycloalkylglycosides, particularly cycloalkylmaltosides (CYMALs). They can be regarded as non-toxic and safe due to their metabolism into CO<sub>2</sub> and water through the corresponding sugar and fatty acid metabolic pathways. These molecules are structurally related to alkylglycosides, which have been used as excipients to promote nasal or ocular absorption and also as stabilizing agents for peptides and proteins.

The chemically synthesized CYMALs are composed of a sugar, typically a disaccharide. The carbohydrate component is linked by an O-glycosidic bond at the "free end" of maltose (carbon atom number 1) to an alkyl chain, wherein the alkyl chain comprises at the most distal position of the alkyl chain, the so-called  $\omega$ -position, a cycloalkyl group. Independently of each other, the preferred disaccharide is maltose; the preferred alkyl chain comprises 1 to 7 methylene groups, i.e. (CH<sub>2</sub>)<sub>1-7</sub>, and the cycloalkyl rest at the  $\omega$ -position is a cyclohexyl group. Thus, the general formula for these compounds reads:  $\omega$ -cycloalkyl-1-(C<sub>1-7</sub>-alkyl)- $\beta$ -D-maltosides, or more specifically  $\omega$ -cyclohexyl-1-(CH<sub>2</sub>)<sub>1-7</sub>- $\beta$ -D-maltosides (= CYMAL<sup>®</sup>). Specific examples of these cyclohexyl alkyl maltosides are 1-cyclohexyl-1-methyl- $\beta$ -D-maltoside (= CYMAL<sup>®</sup>-1), 2-cyclohexylethyl- $\beta$ -D-maltoside (= CYMAL<sup>®</sup>-2), 3-cyclohexyl-1-propyl- $\beta$ -D-maltoside (= CYMAL<sup>®</sup>-3), 4-cyclohexyl-1-butyl- $\beta$ -D-maltoside (= CYMAL<sup>®</sup>-4), 5-cyclohexyl-1-pentyl- $\beta$ -D-maltoside (= CYMAL<sup>®</sup>-5), 6-cyclohexyl-1-hexyl- $\beta$ -D-maltoside (= CYMAL<sup>®</sup>-6), 7-cyclohexyl-1-heptyl- $\beta$ -D-maltoside (= CYMAL<sup>®</sup>-7). Preferred "CYMALs" are CYMAL<sup>®</sup>-5, CYMAL<sup>®</sup>-6 and CYMAL<sup>®</sup>-7, because CYMAL<sup>®</sup>-5, -6 and -7 are easily accessible.

A further group of preferred absorption enhancers are amphiphilic polymers referred to herein as "PMALs", having a highly charged cationic and anionic backbone to which an aliphatic tail is attached. The expression "PMAL" describes alternant polymers prepared by the monomers maleic acid anhydride and an  $\alpha$ -olefine having 6, 8, 10, 12, 14, 16 or 18 carbon atoms, respectively (i.e., "poly(maleic acid anhydride-alt- $\alpha$ -olefine)"), which are grafted with dimethylamino propylamine side chains. They may be prepared by reaction of poly(maleic acid

anhydride-alt- $\alpha$ -olefine) with 3-dimethylamino propylamine, whereby the anhydride group reacts with the primary amino function of the 3-dimethylamino propylamine to form an amide bond. By this way, the "non-reacting" or leaving carboxylic acid function of the original anhydride function is present as a free carboxylic acid, and simultaneously, the basic dimethylamino function is present in the side chain. Thus, a zwitterionic or ampholytic polymer ("amphipol") is obtained. Specific examples of PMALs are alternant polymers of maleic acid anhydride and  $\alpha$ -olefine having 6 to 18 carbon atoms, substituted with 3-dimethylamino propylamine (=PMAL<sup>®</sup>) such as the poly(maleic acid anhydride-alt-1-octene) 3-dimethylamino propylamine derivative (=PMAL-C6<sup>®</sup>); poly(maleic acid anhydride-alt-1-decene) 3-dimethylamino propylamine derivative (=PMAL-C8<sup>®</sup>); poly(maleic acid anhydride-alt-1-dodecene) 3-dimethylamino propylamine derivative (=PMAL-C10<sup>®</sup>); poly(maleic acid anhydride-alt-1-tetradecene) 3-dimethylamino propylamine derivative (=PMAL-C12<sup>®</sup>); poly(maleic acid anhydride-alt-1-hexadecene) 3-dimethylamino propylamine derivative (=PMAL-C14<sup>®</sup>); and poly(maleic acid anhydride-alt-1-octadecene) 3-dimethylamino propylamine derivative (=PMAL-C16<sup>®</sup>). Preferably, poly(maleic acid anhydride-alt-1-dodecene) 3-dimethylamino propylamine derivative (=PMAL-C10<sup>®</sup>) is used.

#### Coating

The coating polymers of the present invention are polyelectrolyte polymers. Suitable polyelectrolyte polymers include an anionic sulfated glycosaminoglycan such as polysaccharide chondroitin sulfate (ChS), and a cationic chitosan derivative such as trimethylchitosan (TMC). These polyelectrolyte polymers have the advantage that they are generally non-toxic and not irritating to the mucosa, and do not require organic solvents for processing them, which would not be desirable from the point of protein stability and additionally adversely affect the patient. ChS is a sulfated glycosaminoglycan composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid). It is usually found attached to proteins as part of a proteoglycan. Due to its natural affinity for proteins, ChS is suitable for its application as protein carrier. Also due to similarity to the structure of mucopolysaccharides, it may have good potential for penetration close to the epithelial wall. TMC may be beneficially used as oppositely charged polymer to further associate with ChS/G-CSF complexes, or with ChS/EPO complexes, and to form nanoparticles providing better protection against protein degradation. It also contributes to the absorption promoting potential of the compositions according to the invention.

#### Nanoparticles

Nanoparticles (NPs) are prepared in aqueous medium under mild conditions which make the method suitable for sensitive protein drugs. Upon stepwise addition of protein, e.g., of G-CSF, or of EPO, and absorption enhancer to the first polymer, followed by slow addition of the second polymer, NPs are formed spontaneously based on the specific interaction between all ingredients. This formation of nanoparticles is primarily driven by the intrinsic physico-chemical properties of both the polymers and the protein; therefore it has to be tailored individually for

each particular protein and polymers selected. The specific properties of the protein, however, play the most important role in the selection of suitable materials and processing conditions for NP-formation.

- 5 The size of the nanoparticles should not exceed a certain threshold. For a suitable formulation with improved muco-adhesiveness, the nanoparticle size should be less than 4000 nanometers, preferably less than 1000 nanometers, more preferably less than 500 nanometers, and most preferably less than 400 nanometers. Nanoparticles with a size from 200 to 400 nanometers are particularly preferred; nanoparticles with a size from 200 to 250 nanometers are more  
10 particularly preferred.

### Example 1

#### Nanoparticles preparation and characterization

- 15 Nanoparticles were obtained following a three step process. Firstly, a buffer solution with G-CSF (4.4 mg/ml in 10 mM acetate buffer with 5% sorbitol, pH 4.5, 0.23 ml) was added in a drop wise manner into aqueous solution of permeation enhancer CYMAL-7 (6-cyclohexyl-1-heptyl-maltoside; 15 mg/ml, 0.1 ml) under gentle stirring (15 min) at room temperature. The mixture was then slowly added into a chondroitin sulfate solution (ChS, 2 mg/ml in 0.25 % acetic acid,  
20 pH 3.0, 2.0 ml). After gentle stirring for 20 min, trimethylchitosan solution (TMC, 1 mg/ml in 0.25 % acetic acid, pH 3.5, 1.8 ml) was added to the final dispersion and stirred for additional 60 min for complete nanoparticle recovery. Nanoparticles had a uniform size of around 200-250 nm with low polydispersity (Pdl ~ 0.2) and zeta potential -26 mV (Zetasizer NanoZS, Malvern Instruments). Scattering intensity (measured as derived count rate) increased remarkably from  
25 360 kcps for polymer solution to 220,000 kcps for the final dispersion reflecting efficient NP formation with characteristic dispersion turbidity.

- Freshly prepared nanoparticles were analyzed for protein loading. The solution of nanoparticles was centrifuged using an ultracentrifuge at 40.000 rpm for 20 minutes. The G-CSF concentration  
30 in the supernatant was determined using RP-HPLC chromatography and calculated according to the G-CSF standard with known concentration. The association efficiency of G-CSF in NPs was between 70 % and 85 % resulting in final G-CSF loading in NPs between 8.5 and 10.3 % of NPs mass.

- 35 Release studies were performed at pH 5.8, which is the expected pH in the intestine. Two doses of formulation (2x 0.5 ml of NPs dispersion containing 2x 0.125 mg G-CSF) were mixed with phosphate buffered saline (pH 6.0) till the final volume of 5 ml. pH was adjusted to 5.8 by the addition of 0.2 M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub>. Formulations were incubated at 37 °C with constant shaking. At different time points samples were collected and centrifuged at 13.000 rpm for 20 minutes.  
40 The G-CSF concentration in supernatant was determined using RP-HPLC chromatography and

calculated according to the G-CSF standard with known concentration. The amount of released G-CSF was around 40 % after 1 hour and reached 60 % after 4 hours.

The *in vitro* biological activity of released G-CSF was determined by proliferation assay using NFS-60 cells. Cells were grown in RPMI 1640 medium with 10% FBS (100 % relative humidity, 37 °C and 5 % CO<sub>2</sub>). One day prior to testing cells were transferred to fresh medium, and the next day the cells were washed three times with RPMI 1640 medium with 10% FBS and seeded to 96-well microtiter plates at a density of  $1 \times 10^4$  cells per well. Cells were treated with G-CSF standard and samples in serial dilutions. The plates were incubated at 37°C in a 5 % CO<sub>2</sub> incubator for 48 h. The proliferative response was determined by addition of 20 µl of Presto blue reagent (Molecular Probes). After 3 hours incubation the fluorescence of resulting product was measured. The *in vitro* biological activity was calculated using the PLA-1.1 program (Stegmann Systems). Results showed that G-CSF released from nanoparticles fully retained its biological activity.

15

## Example 2

### *In vivo* studies

All *in-vivo* experiments were performed in female Wistar rats (RccHan<sup>TM</sup>:WIST), weighing 200 – 240 g. The test items were administered by a single intraduodenal (i.d.) administration to groups of 6 rats each. Test items were: (i) G-CSF-loaded nanoparticles of the present invention, (ii) placebo – empty nanoparticles having the same composition as NPs of the present invention but without the protein G-CSF, and (iii) solution of free G-CSF in buffer solution.

Prior to the application, the rats were fasted for about 6 hours. For i.d. administration, the rats were anaesthetized with intraperitoneal injection of a mixture of ketamine, xylazine and saline (5:4:5 v/v/v; 0.5 ml/rat, 20 % w/v solution). An ocular lubricant Bivacyn was applied to the animal's eyes. The incision site at abdominal region (region xiphoides) was shaved and disinfected with an iodine solution. The rat was then positioned on a heated sterile operation cover and covered with a sterile drape. The abdomen was entered through a midline incision, and the duodenum was located. The upper part of the duodenum was gently lifted. A UV sterilized Latex Free syringe with 23G needle was introduced into the duodenum, and protease inhibitor cocktail (Sigma) was administered into the duodenum. The test items were applied through the same needle. The test items containing G-CSF were administered at dose of 125 µg/rat and the volume of administration of all test items was 0.5 ml. The injection site was gently pressured after application to stop any leaking or bleeding. The duodenum was carefully returned to its normal position within the abdominal cavity. The abdominal muscles were closed with a 6-0 absorbable suture and the abdominal skin was closed with the wound clips. The incision site was sprayed with Bivacyn. Each rat received 0.03 ml of the antibiotic Trioxyl intramuscularly. After surgery, the rats were caged individually and placed in a warm room (T = 24 ± 1 °C) for at least 3 h or until complete recovery from anesthesia.

Blood samples for determination of absolute neutrophil count were collected from the terminal tail vein before the application and 12, 24, 48, 72, 144 and 168 hours after application. The samples of blood (30 µl) were taken directly from the vein into the pipette and immediately  
5 diluted with Cellpack reagent, Sysmex (120 µl; 1/5, v/v). The diluted samples were gently mixed and immediately analyzed with the hematological analyzer Sysmex XT-2000iV. The following hematological parameters were analyzed: red blood cell count, white blood cell count, hemoglobin, Hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, platelets and differential leukocyte count: neutrophils, lymphocytes, eosinophils,  
10 basophils and monocytes.

The evaluation of the effect was performed in terms of changes in the number of peripheral neutrophilic granulocytes (Figure 1 and 2). The average neutrophil count at each time point after intraduodenal administration was plotted against time. Data from the G-CSF-loaded NPs of the  
15 present invention as well as for the G-CSF solution was compared with corresponding control-placebo group by the t-test with significant level of  $P < 0.05$ .

All intraduodenally administered items including placebo showed a peak in neutrophil count approximately 12 hours post application (Figure 1 and 2).

20

The neutrophils increased 4.6-fold in the placebo group and 8.6-fold in the group receiving G-CSF-loaded NPs as compared to the pre-dose level (Figure 1).

The statistical analysis of the neutrophil increase in that time point (12h) showed that the difference in the neutrophil increase between G-CSF-loaded NPs and placebo is statistically  
25 significant.

The increase in neutrophil count in placebo group indicates that operation procedure and application into the duodenum slightly affect the neutrophil count in that time point (12h). However, significantly greater increase in the number of neutrophils in the animal group  
30 receiving G-CSF-loaded NPs clearly indicates that this neutrophil increase not only results from operation and application procedure but reflects also the effect of G-CSF delivered by NPs and being absorbed from the intestine.

The results of G-CSF in solution showed that the neutrophils increased 5.5-fold after 12 hours compared to the pre-dose level (Figure 2). The statistical analysis of G-CSF free in solution and placebo showed that the difference in the point 12h is not statistically significant.

To conclude, substantially higher pharmacodynamic response of intraduodenally administered G-CSF was obtained for G-CSF-loaded NPs of the present invention in comparison to G-CSF  
40 solution.

### Pharmacological availability

The pharmacological availability of intraduodenally administered G-CSF formulation relative to subcutaneously administered G-CSF was calculated by utilizing the equation:

$$PA_{i.d.} \% = \frac{AUC_{i.d.(0 \rightarrow 48h)}}{AUC_{s.c.(0 \rightarrow 48h)}} \times \frac{Dose_{s.c.}}{Dose_{i.d.}} \times 100$$

where  $PA_{i.d.}$  is relative pharmacological availability of intraduodenally administered G-CSF-loaded NPs,  $AUC_{i.d.(0 \rightarrow 48h)}$  and  $AUC_{s.c.(0 \rightarrow 48h)}$  are the areas under the curves (pharmacological responses) of intraduodenally administered G-CSF-loaded NPs and subcutaneously given G-CSF solution, respectively, and  $Dose_{i.d.}$  and  $Dose_{s.c.}$  are the doses of G-CSF administered in NPs intraduodenally (125 µg/rat) and G-CSF solution administered subcutaneously (1 µg/rat or 10 µg/rat), respectively.

The pharmacological response of subcutaneously administered G-CSF solution at doses 1 µg/rat and 10 µg/rat is shown in Figure 3.

AUC was calculated for time points from 0 to 2 days, since the peak in the neutrophil increase occurred 12 hours after enteral application of G-CSF-loaded NPs or 6 hours after subcutaneous administration of G-CSF solution.

$AUC_{i.d.(0 \rightarrow 48h)}$  that was taken into account in the calculation of  $PA_{i.d.}$  represents the difference between AUC obtained for intraduodenally administered G-CSF-NPs and AUC obtained for intraduodenally administered placebo – empty NPs. The effect of the surgical procedure on neutrophil count was therefore eliminated (see figure 1).

$AUC_{s.c.(0 \rightarrow 48h)}$  that was taken into account in the calculation of  $PA_{i.d.}$  represents the AUC obtained for subcutaneously administered G-CSF solution (1 µg/rat or 10 µg/rat) from which the basal level in neutrophil count was subtracted.

The pharmacological availability of intraduodenally administered G-CSF (125 µg/rat) loaded in NPs was calculated relative to subcutaneously injected G-CSF solution given at two different doses, 1 µg/rat or 10 µg/rat.

The pharmacological availability of intraduodenally administered G-CSF (125 µg/rat) loaded in NPs relative to subcutaneously injected G-CSF solution at doses of 1 µg/rat and 10 µg/rat was 1.3% and 2.5%, respectively.

**Claims**

1. Composition comprising a protein, at least one absorption enhancer and at least two oppositely charged polyelectrolyte polymers, wherein the absorption enhancer is a cycloalkylglycoside.
2. The composition of claim 1, wherein the protein is selected from the group of a G-CSF, a human G-CSF, a recombinant human G-CSF (rhG-CSF), an EPO, a human EPO, and a recombinant human erythropoietin (rhEPO).
3. The composition of claims 1 or 2, wherein the protein is a human G-CSF.
4. The composition of any one of the preceding claims, wherein the cycloalkylglycoside is a cycloalkylmaltoside.
5. The composition of any one of the preceding claims, wherein the cycloalkylglycoside is CYMAL<sup>®</sup>-5, CYMAL<sup>®</sup>-6 or CYMAL<sup>®</sup>-7, or wherein the cycloalkylglycoside is CYMAL<sup>®</sup>-7.
6. The composition of any one of the preceding claims, wherein one of the at least two polyelectrolyte polymers is a sulfated glycosaminoglycan.
7. The composition of any one of the preceding claims, wherein one of the at least two polyelectrolyte polymers is a chitosan derivative.
8. The composition of claims 6 or 7, wherein the sulfated glycosaminoglycan is chondroitin sulfate.
9. The composition of claims 7 or 8, wherein the chitosan derivative is trimethylchitosan.
10. The composition of any one of the preceding claims, comprising a protein, a cycloalkylmaltoside, chondroitin sulfate and trimethylchitosan, or comprising a protein, CYMAL<sup>®</sup>-7, chondroitin sulfate and trimethylchitosan.
11. The composition of claim 10, wherein the protein is a human G-CSF, or a human EPO, preferably a human G-CSF.
12. The composition of any one of the preceding claims having a particle size of less than about 4000 nanometers, of less than about 1000 nanometers, of less than about 500 nanometers, or of less than about 400 nanometers, and preferably of from about 200 to about 400 nanometers, or from about 200 to about 250 nanometers.
13. The composition of any one of the preceding claims for use in therapy or diagnosis.
14. Use of the composition of any one of the preceding claims for the preparation of a medicament.
15. The composition of any one of claims 1 to 14 for use in the reduction of the duration of neutropenia, in the reduction of the duration of severe neutropenia and its associated complications, or in the mobilization of peripheral blood progenitor cells.

16. A process for preparing a pharmaceutical composition according to any one of claims 1 to 12, comprising the steps of
- (a) adding a solution of protein to a first solution comprising at least one absorption enhancer to give a first mixture,
- 5 (b) adding said first mixture to a solution comprising a first polyelectrolyte polymer to give a second mixture,
- (c) adding a solution comprising a second polyelectrolyte polymer to said second mixture, and (d) isolating the resulting nanoparticles;
- 10 wherein the first polyelectrolyte polymer and the second polyelectrolyte polymer are oppositely charged, and wherein the absorption enhancer is a cycloalkylglycoside.
17. The process of claim 16, wherein the at least one absorption enhancer is CYMAL<sup>®</sup>-5, CYMAL<sup>®</sup>-6 or CYMAL<sup>®</sup>-7, or wherein the at least one absorption enhancer is CYMAL<sup>®</sup>-7.
18. The process of claims 16 or 17, wherein the first polyelectrolyte polymer is chondroitin sulfate.
- 15 19. The process of any one of claims 16 to 18, wherein the second polyelectrolyte polymer is trimethylchitosan.
20. The process of any one of claims 16 to 19, wherein the protein is a human G-CSF, or a human EPO, preferably a human G-CSF.
21. The composition of any one of claims 1 to 12 and 15, or the use of claim 14, or the
- 20 process of any one of claims 16 to 20, wherein the composition is an oral composition.

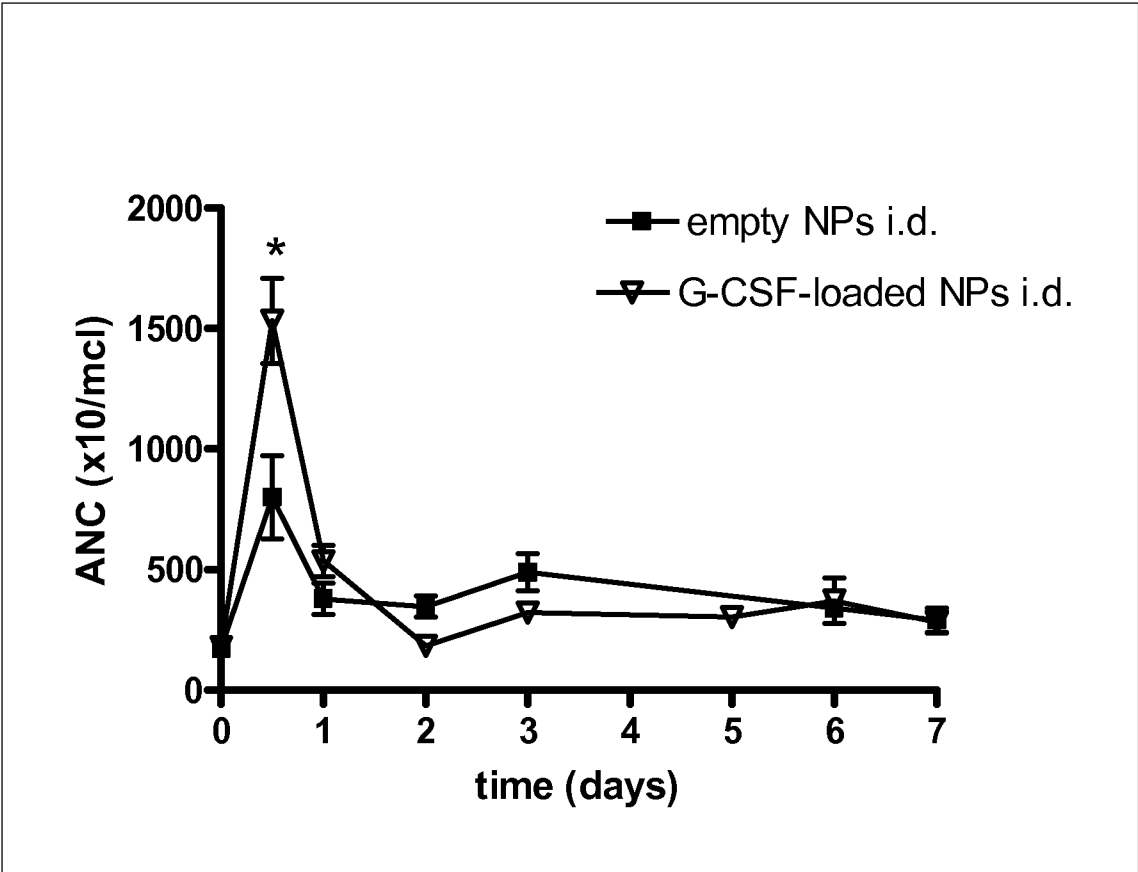


Figure 1

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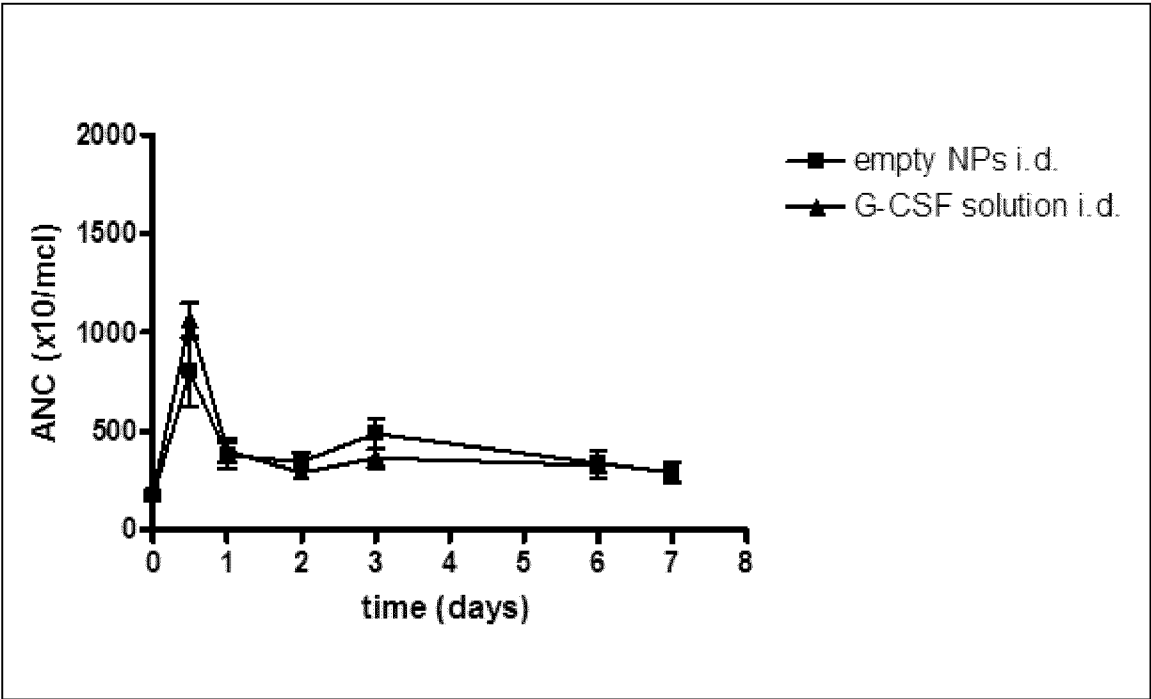


Figure 2

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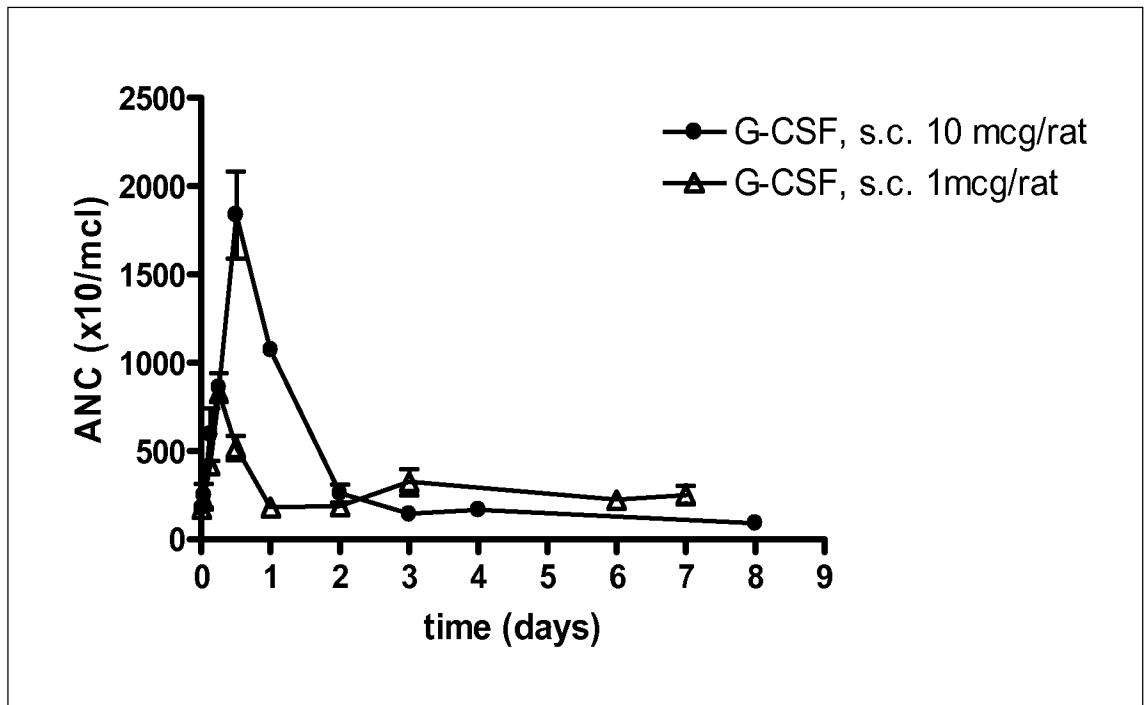


Figure 3

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/069181

## A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K9/51 A61K38/19  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                     | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y         | <p>AMIDI M ET AL: "Chitosan-based delivery systems for protein therapeutics and antigens",<br/>ADVANCED DRUG DELIVERY REVIEWS, ELSEVIER,<br/>vol. 62, no. 1,<br/>31 January 2010 (2010-01-31), pages 59-82,<br/>XP026877915,<br/>ISSN: 0169-409X<br/>[retrieved on 2009-11-16]<br/>abstract<br/>page 67<br/>page 73, left-hand column, lines 8-21<br/>item 6.1.1</p> <p style="text-align: center;">-----<br/>-/--</p> | 1-21                  |



Further documents are listed in the continuation of Box C.



See patent family annex.

## \* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

21 November 2014

Date of mailing of the international search report

09/12/2014

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## INTERNATIONAL SEARCH REPORT

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| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                          |                       |
|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                       | Relevant to claim No. |
| Y                                                    | WO 2011/163458 A2 (GENENTECH INC [US];<br>HOFFMANN LA ROCHE [CH]; ESUE OSIGWE [US];<br>SHARMA V) 29 December 2011 (2011-12-29)<br>claims 1,3<br>page 36, paragraph first<br>page 2, lines 21-24<br>----- | 1-21                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/069181

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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