AUG 2

Supplementary Information for

Synthesis and Characterization of Heparosan-Granulocyte-Colony Stimulating Factor Conjugates: a natural sugar-based drug delivery system to treat neutropenia

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Supplementary Table

Treatment Groups	Fold Increase					
	WBCs	Absolute Neutrophils	Absolute Lymphocytes	Absolute Monocytes	Absolute Basophils	Absolute Eosinophils
	Days 2-3	Days 2-8	Days 2-3	Days 2-6	Days 2-3	Days 2-3
20-kDa PEG-GCSF	+1.6–1.7	+0.10–7.9	+0.14-0.29	+0.17–4.7	+2.0-2.4	+1.0-1.1
55-kDa HEP-GCSF	+1.3–1.4	+0.07–5.4	+0.17-0.44	+0.50–3.8	+1.5-2.0	+0.24-1.7
99-kDa HEP-GCSF	+1.2–1.3	+0.41–5.7	+0.16-0.22	+0.61–4.0	+1.3-1.7	+0.53-1.9
Neulasta	+1.8–2.1	+0.30–8.9	+0.32-0.62	+0.36–5.0	+2.6-3.7	+0.61-1.2

Supplementary Table 1: Summary of hematological changes in rats injected with G-CSF conjugates.

The fold-increase is listed above for various blood cell types at various times post-injection in comparison to the buffer vehicle alone. (*n* = ~5/group; values represents change from concurrent control on the given study period time).

Supplementary Figures

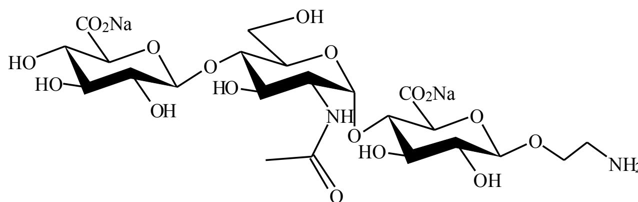
Supplementary Figure 1. Structure of Acceptor and HEP derivatives

First, the heparosan polymer was produced by synchronized stoichiometrically controlled polymerization using a heparosan trisaccharide amine-containing glycoside acceptor (**A**) and UDP-GlcA and UDP-GlcNAc with the HEP synthase. This HEP-NH_2 polymer (n = ~145 or ~260 for the 55- or 99-kDa polymers, respectively) was purified. In the second step, HEP-NH_2 was converted to the aldehyde form via reaction with a heterobifunctional reagent to create HEP-CHO (**B**).

The HEP-CHO and G-CSF were coupled by reductive amination at pH 5 to more selectively react with the amino terminus of the protein (rather than lysine side-chains) yielding the HEP-G-CSF conjugate (**C**). To define the coupling site, the conjugate was first treated with heparin lyase III (cleaves the sugar chain, leaving an unsaturated glucuronic acid derivative at the non-reducing end) and the protein with a 3-sugar unit 'stub' was PAGE purified. Second, the relevant gel slice was excised and treated with the protease trypsin to generate an oligosaccharide-peptide species (**D**) that was identified by liquid chromatography/mass spectrometry (*see* **Supp. Fig. 5**).

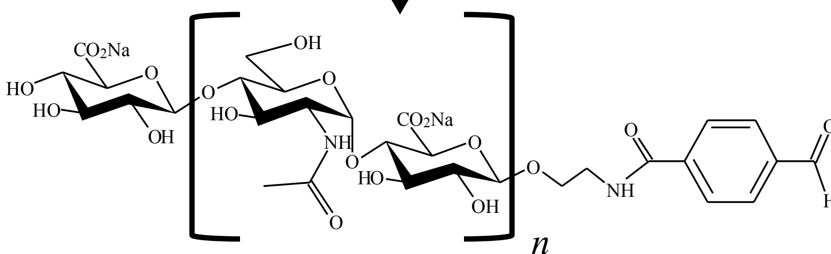
Supplementary Fig. 1

A.



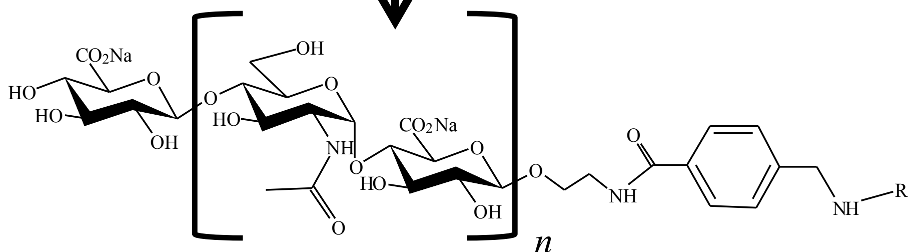
1. PmHS1, UDP-sugars
2. succinimidyl-*p*-formylbenzonate

B.



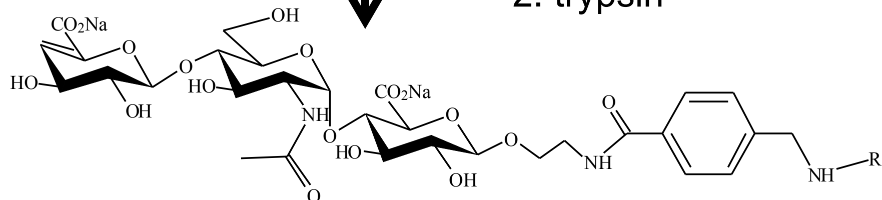
G-CSF, $NaCNBH_3$, pH 5.0

C.



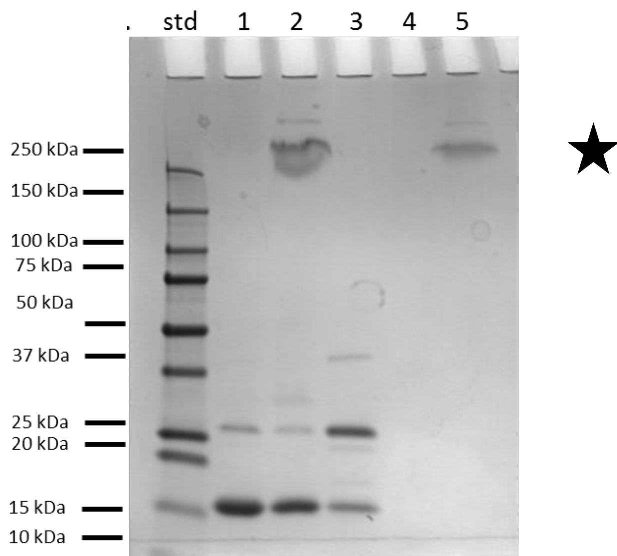
1. Hep lyase III
2. trypsin

D.

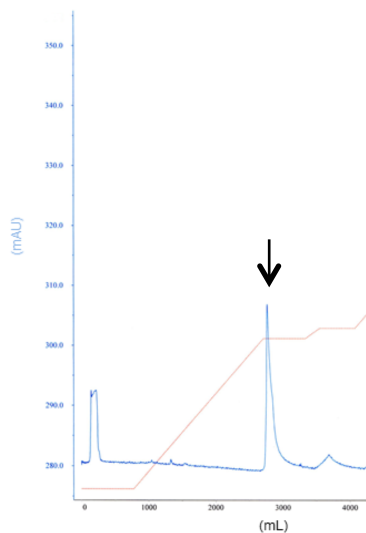


R_1 = G-CSF
R_2 = amino-terminal tryptic peptide

a



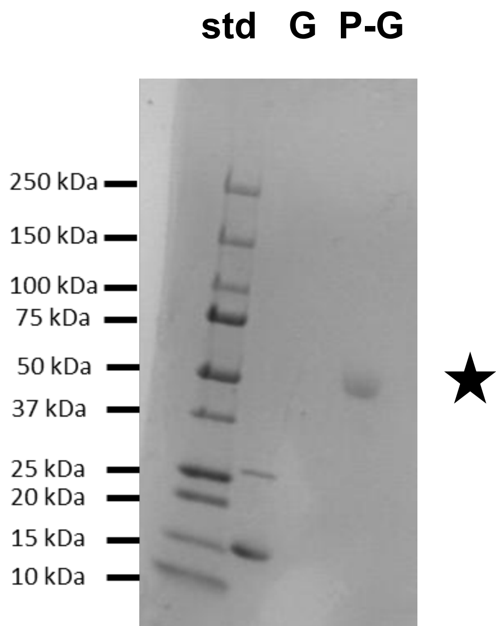
b



Supplementary Figure 2. Electrophoretic analyses of 99-kDa HEP-G-CSF and strong anion exchange (SAX) chromatography purification of 55-kDa HEP-GCSF.

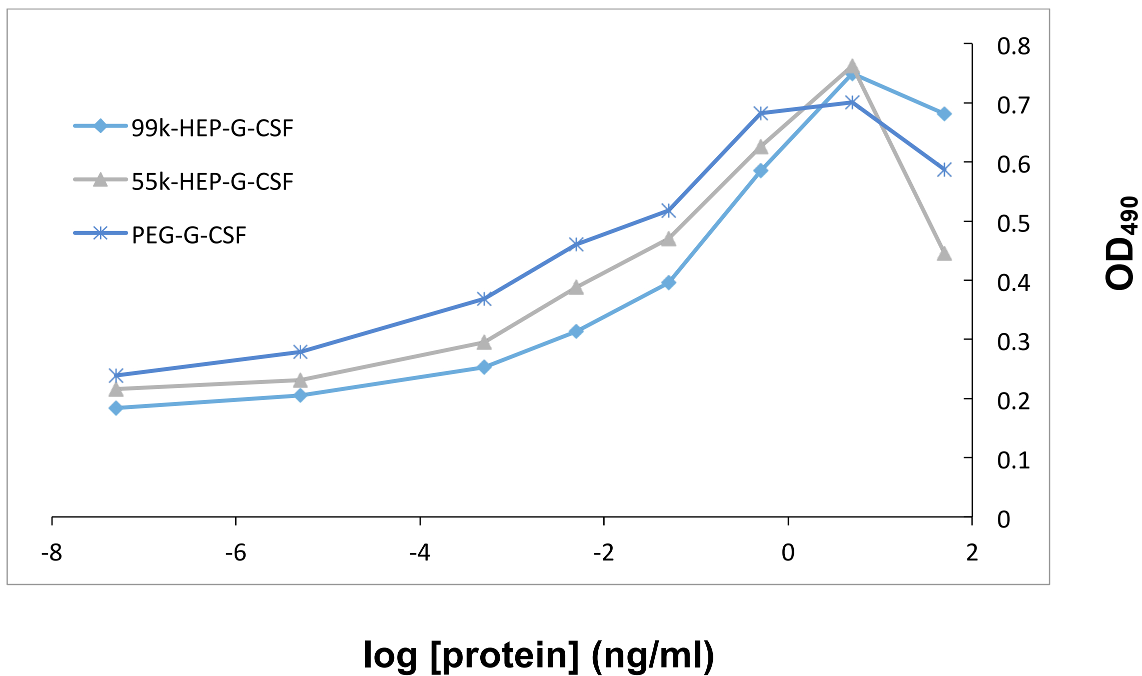
Panel *a* - Samples were separated in 4-20% TGX gradient gel and stained with Coomassie Blue. **std**, Precision Plus® protein size standard. **Lane 1**, free G-CSF. **Lane 2**, conjugation reaction mixture before purification. **Lane 3**, uncoupled protein in the flow-through of SAX. **Lane 4**, blank. **Lane 5**, purified HEP-G-CSF conjugate (marked with a *star*).

Panel b - SAX profile of the 55-kDa-HEP-G-CSF purification depicted in Fig. 2 of main text. The peak marked with the *arrow* was pooled and used for animal efficacy testing (*blue*, UV absorbance at 215 nm; *orange*, NaCl gradient).



Supplementary Fig 3. SDS-PAGE analysis of purified PEG-G-CSF.

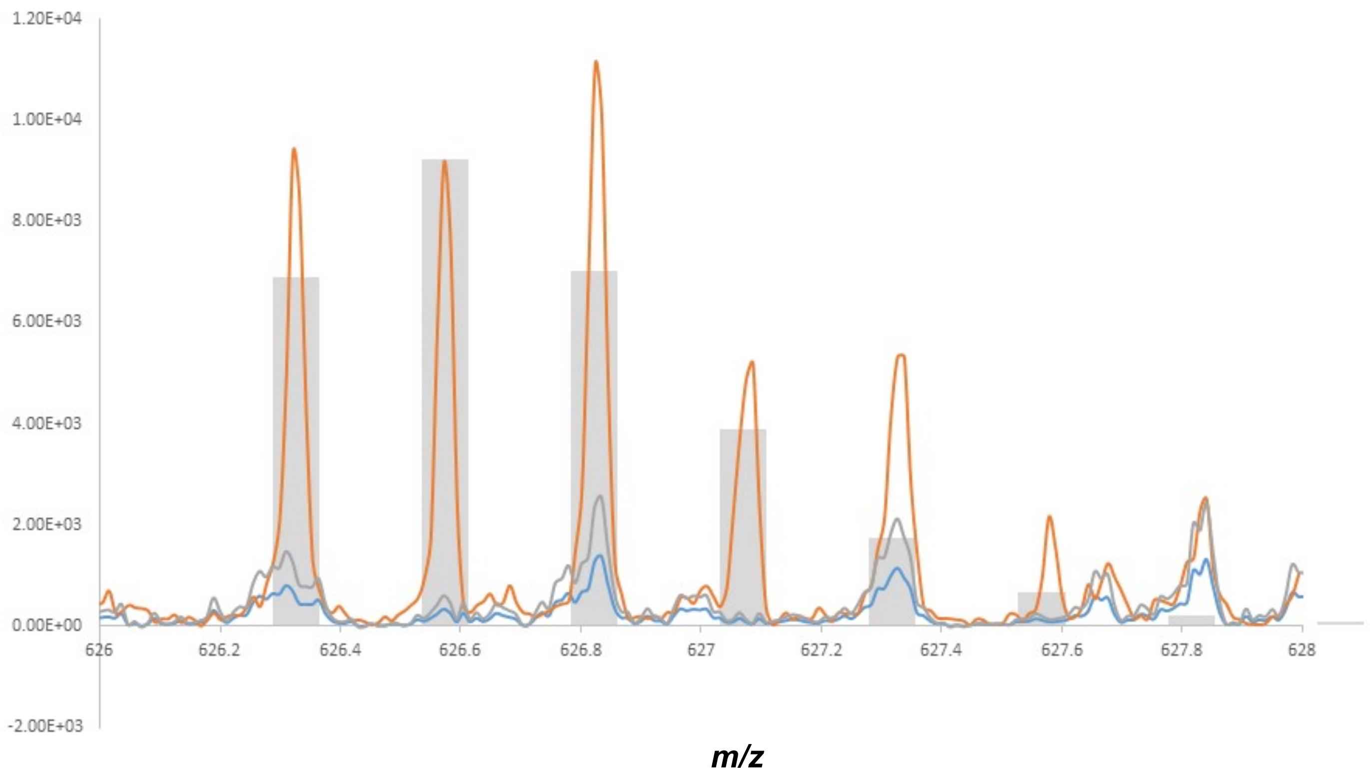
The starting protein and the in-house prepared, cation exchange chromatography-purified PEG-conjugate were analyzed on 4-20% TGX gel with Coomassie Blue staining. **Lanes: G**, G-CSF; **P-G**, PEG-G-CSF (*star*); **std**, Precision Plus® protein standard.



Supplementary Fig 4. NFS-60 cell proliferation assay of 55-kDa and 99-kDa HEP-G-CSF and PEG-G-CSF.

All 3 conjugates stimulated proliferation in culture. The approximate effective concentration for 50% effect (EC_{50}) values (pg/ml) were: PEG-G-CSF, 4.1; 55-kDa HEP, 20; 99-kDa HEP, 25.

Intensity

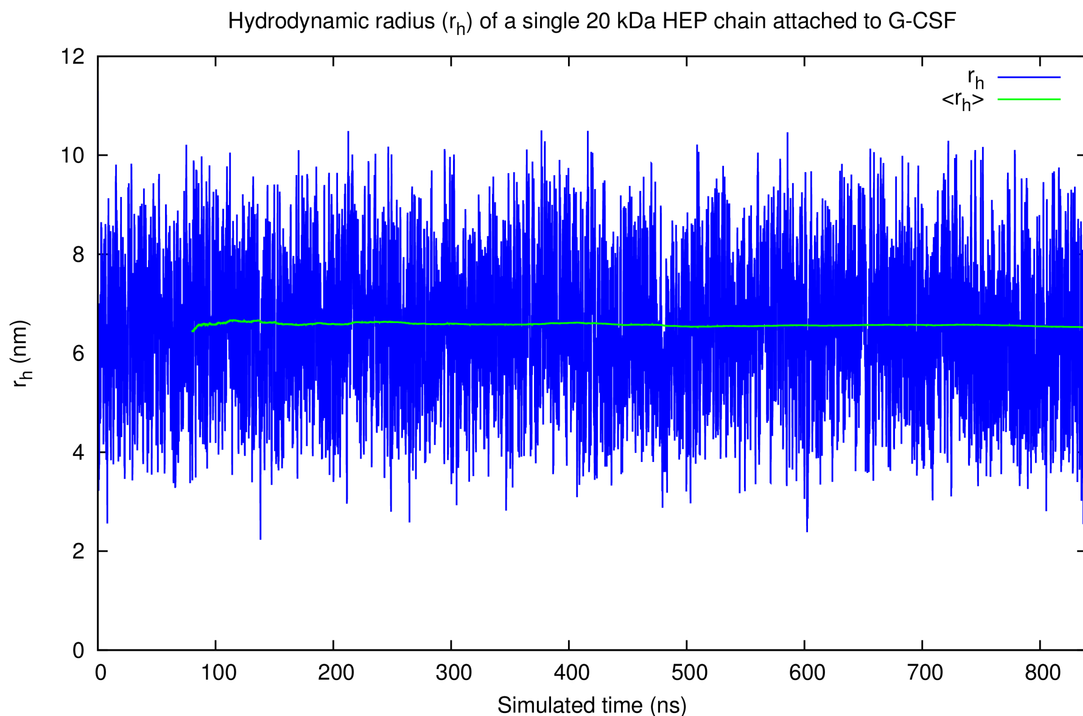


Supplementary Figure 5. LC-MS-MS analysis of trisaccharide-peptide 'stub' of HEP-G-CSF conjugate.

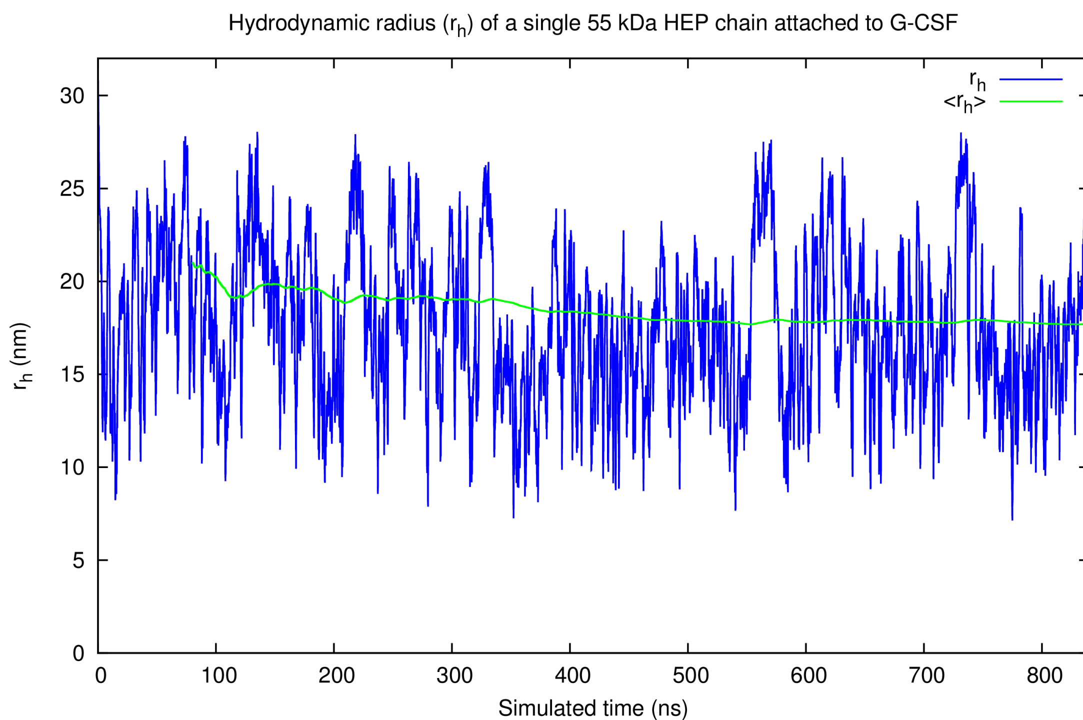
The 55-kDa HEP modified G-CSF (*orange*) or unmodified protein (*blue; grey* normalized to HEP-G-CSF intensity for other peptide species) were analyzed in parallel by peptide mapping. A unique +4-charged species (composed of MTPLGPASSLPQSFLLK: $C_{82}H_{136}N_{19}O_{23}S_1$ and Hep3-linker: $C_{30}H_{40}N_2O_{18}$ forming the complex: $C_{112}H_{174}N_{21}O_{41}S_1$; standard formula weight: 2502.761; *see* Supp. Fig. 1, structure **D**) was observed confirming coupling of the heparosan chain to the amino-terminus of the protein drug.

Supp. Fig 6. Molecular simulation data for hydrodynamic radius predictions of HEP-G-CSF conjugates.

The data below for the 20-kDa, 55-kDa, or 99-kDa HEP-G-CSF was used to generate the values in **Table I**; the *green trace* reflects the average r_h over time (cumulative moving average). Note that the longer sugar chains displayed a wider range of possible configurations and consequently the same number of computations resulted in a less accurate average r_h prediction.

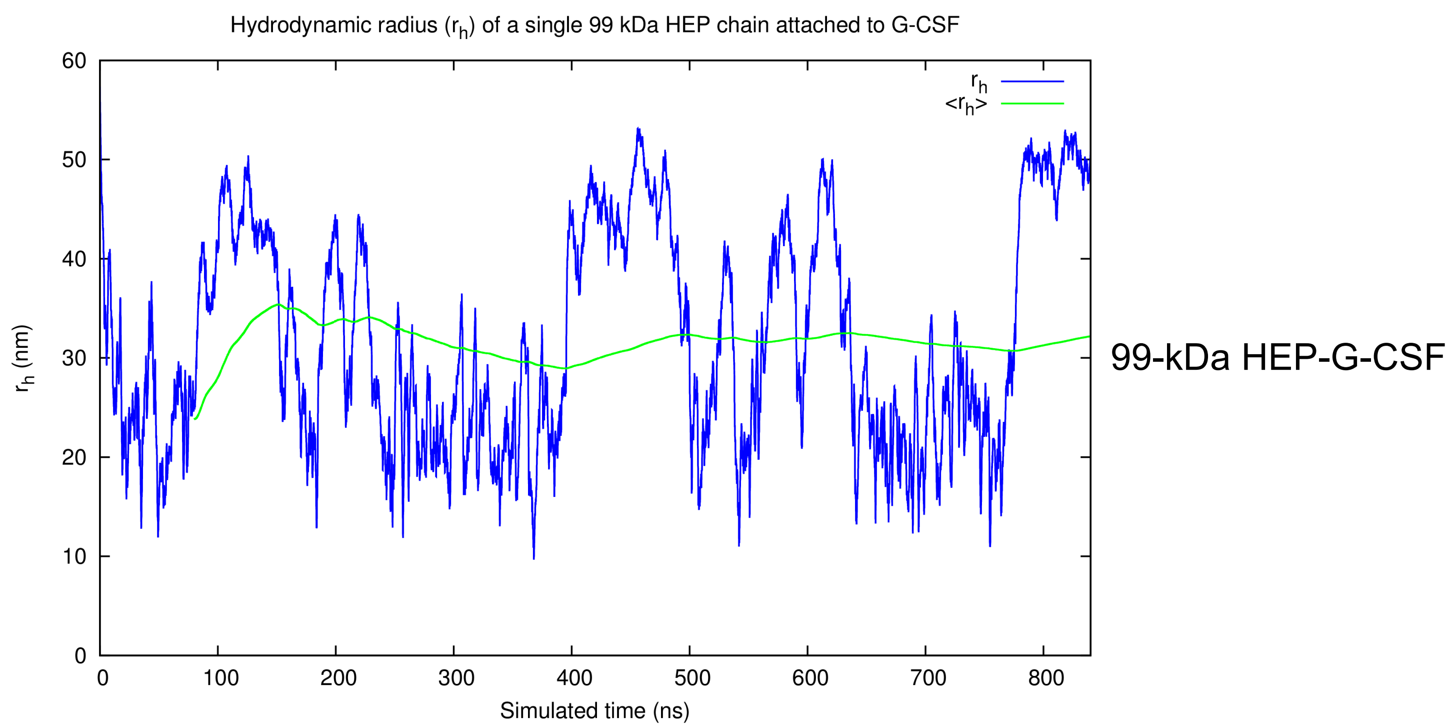


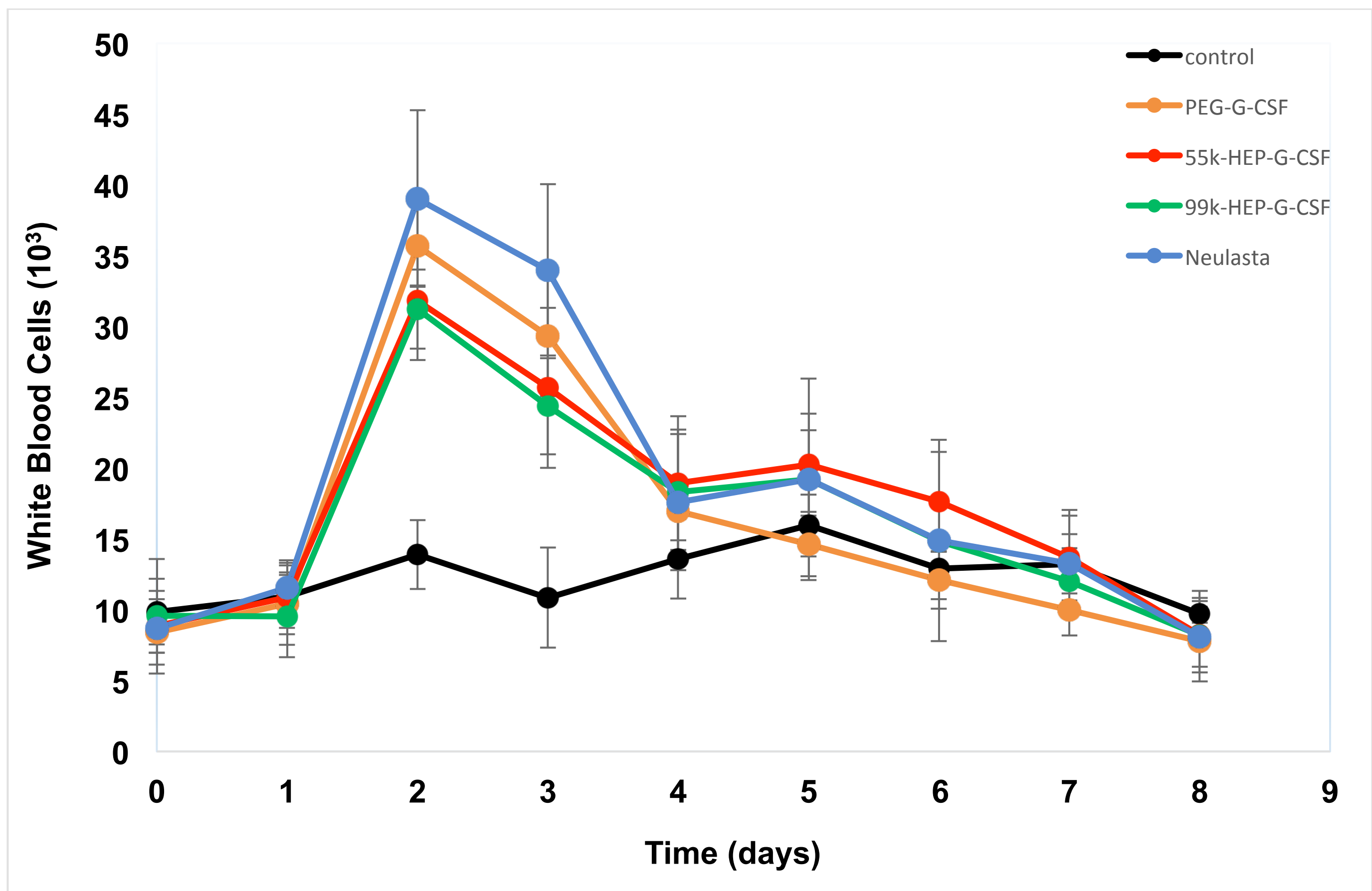
20-kDa HEP-G-CSF



55-kDa HEP-G-CSF

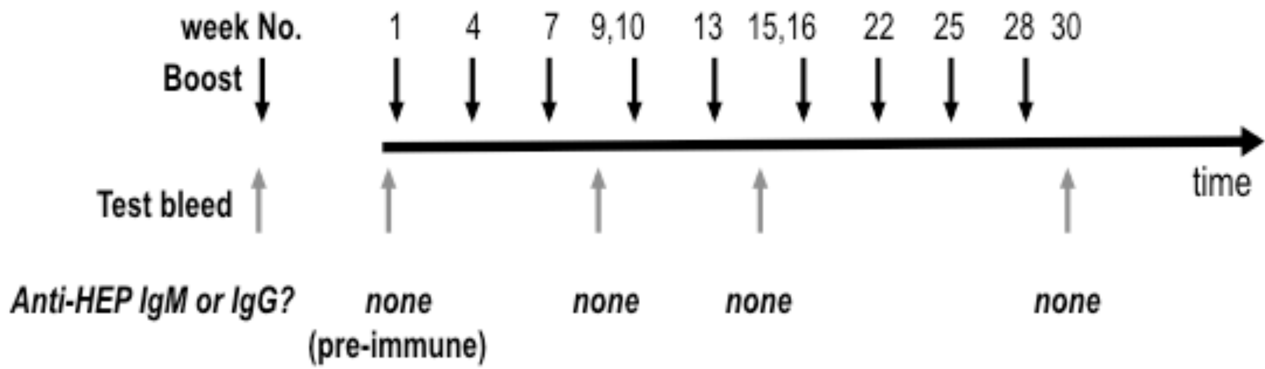
Supp. Fig 6. Molecular simulation data for hydrodynamic radius predictions of HEP-G-CSF conjugates. (cont.)





Supplementary Figure 7. Hematology study with single injection of HEP-G-CSF (55- or 99-kDa), PEG-G-CSF (in-house prepared), or Neulasta® in healthy rats.

The absolute white blood cell counts were stimulated by all conjugates in comparison to the vehicle control (*black*).



Supplementary Figure 8. **Immunization and bleeding schedule of the long-term immunological study in three Sprague Dawley rats.**

The rats were boosted with 55-kDa HEP-G-CSF antigen nine times approximately every three weeks. *Arrows* indicate the time of antigen boost (*black*) and bleed collection (*gray*). Each rat received 55-kDa HEP-G-CSF about every three weeks at 0.4-0.7 mg/kg. Serum were collected before the first injection (week zero = ‘prebleed’) and at week 9, 15 and 27 (rat #3) or at week 9, 15 and 30 (rat #1, and #2) (post-injection bleed 1, 2, and 3). To detect potential anti-heparosan antibodies, enzyme-linked immunosorbent assay (ELISA) plates were coated with HEP-BSA (a conjugate with bovine serum albumin). As a negative control, some wells were treated with BSA only. The presence of any potential anti-HEP-G-CSF antibodies was analyzed by ELISA with goat anti-rat IgG or IgM as the secondary antibodies (as horseradish peroxidase conjugates; *see* Fig. 5 for results).