

Evaluation of Immunostimulatory Potential of Branded and US-Generic Enoxaparins in an In Vitro Human Immune System Model

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Abstract

Low-molecular-weight heparins (LMWHs) have several positive therapeutic effects and can also form immunostimulatory complexes with plasma proteins, such as platelet factor 4 (PF4). We compared the innate response and functional profiles of branded and US-generic enoxaparins from 2 manufacturers in either native or PF4-bound forms in an in vitro model of human immunity. In an analysis of 2 product lots from each manufacturer and multiple separate batches of protein–heparin complexes, branded enoxaparin was shown to be consistently nonstimulatory for innate responses, whereas US-generic enoxaparins generated variable immunostimulatory profiles depending on the enoxaparin lot used to prepare the PF4–LMWH complexes. Production of tissue factor pathway inhibitor (TFPI), a physiologic heparin-induced inhibitor of tissue factor-induced coagulation that was used as a functional readout of biological activity of enoxaparins in these assays, was heightened in the presence of branded enoxaparin complexes, but its levels were variable in cultures treated with complexes containing US-generic enoxaparins. Analytical analyses suggest that the heightened immunostimulatory potential of some of the US-generic enoxaparin product lots could be tied to their capacity to form ultra-large and/or more stable complexes with PF4 than the other LMWHs included in this study. Although these distinct biological and analytical profiles might be related to the composition and/or consistency of branded and US-generic enoxaparins included in our data set, further studies are warranted to elucidate the pathophysiological relevance of these in vitro findings.

Keywords

heparin, low-molecular-weight heparins, enoxaparin, platelet factor 4, human immunity

Introduction

Heparin is widely used as an injectable anticoagulant for the prevention and treatment of thromboembolism and cardiovascular disorders.¹ One of its primary modes of action toward achieving these clinical benefits is to significantly enhance, by about 1000-fold, the activity of the endogenous coagulation inhibitor, antithrombin, against key coagulation proteases.² As a complementary action, heparin also triggers the release of the anticoagulant factor, tissue factor pathway inhibitor (TFPI), by endothelial cells.^{2,3}

Heparin is not a single molecular entity; rather, it represents a complex mixture of naturally occurring glycosaminoglycans comprising variable-length chains of repeating disaccharides with irregular sulfation patterns.^{4,5} Variations in the molecular composition of heparins have been shown to play an important role in determining the functional activity (protein-binding characteristics) of these moieties.^{6,7} For example, the binding of heparin to antithrombin relies on the glycosaminoglycans

containing specific pentasaccharide sequences. In a second example, the binding of heparin to other positively charged proteins, such as platelet factor 4 (PF4), appears to be impacted by the polysaccharide molecular weight and charge density (number and position of sulfate groups).⁸

Observations regarding the protein-binding characteristics of this anticoagulant have important implications for the production of injectable heparins because some protein–heparin aggregates, including PF4–heparin complexes, have been shown to trigger antibody responses⁹⁻¹¹ and activate dendritic

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Table 1. List of Heparins and PF4–Heparin Complexes Used in the Study.

Heparin Type:		PF4 Complexes Prepared With										
		UFH	ULMWH	Branded Enoxaparin (Sanofi)		US Generic Enoxaparin (Sandoz)			US Generic Enoxaparin (Amphastar)			
Heparin Lot:		Lot 1 (ES 1037-2518)	Lot 1 (P-3308 1-076.1)	Lot 1 (EEA 1412E)	Lot 2 (EEB1402)		Lot 1 (917499)	Lot 2 (917999)		Lot 1 (EP 002B2)	Lot 2 (EP099J1)	
PF4-Heparin Batch:		Batch 1	Batch 1	Batch 1	Batch 1	Batch 2	Batch 1	Batch 1	Batch 2	Batch 1	Batch 1	Batch 2
Study Phase	Pilot Study:	✓	✓	✓								
	Blinded Study:	✓		✓	✓		✓	✓		✓	✓	
	Follow-up Study:	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓

Abbreviations: UFH, unfractionated heparin; ULMWH, ultra low-molecular-weight heparin; PF4, platelet factor 4.

cells (DCs).¹² Based on the average smaller size of the sugar moieties in pharmaceutical-grade, low-molecular-weight heparin (LMWH), it might be anticipated this heteropolymer would have a reduced capacity to engage the immune system since it should less efficiently bind PF4 and other positively charged proteins.^{13,14} However, this premise is complicated by the fact that manufactured LMWHs can have unique physicochemical properties (polysaccharide length and structure, sulfation degree and pattern, and level of impurities) resulting from disparities in the source of biological material used to derive the heparins and/or differences in the protocols used to depolymerize the native heparins into LMWHs that could also affect the protein-binding/immunostimulatory potential of these products.¹⁵

To examine whether distinctions in enoxaparin manufacturing processes impact their capacity to trigger immune reactions, we used an *in vitro* model of the human immune system, termed the Modular Immune *In vitro* Construct (MIMIC[®]),¹⁶ to evaluate innate responses induced by distinct lots of protein–heparin complexes prepared with PF4 and branded and US-generic enoxaparins.¹⁷ In addition to immune readouts, TFPI secretion was used as a functional assessment of LMWH activity in the MIMIC[®] system because it is a crucial pharmacodynamic marker of heparin under physiologic conditions.¹⁸ Finally, analytical assessments, including native gel electrophoresis and size exclusion fast performance liquid chromatography (SE-FPLC), were performed to evaluate the capacities of the branded and US-generic enoxaparins to form and maintain large/stable complexes with PF4.

Materials and Methods

Study Design

This 3-phase study was designed to evaluate the innate stimulatory profiles of branded enoxaparin and 2 United States-approved generics manufactured by Sandoz (Princeton, NJ)

and Amphastar (Rancho Cucamonga, California).¹⁷ The first study phase consisted of pilot experiments in which complexes of PF4 with unfractionated heparin (UFH), branded enoxaparin, and ultra-LMWH (ULMWH) were tested for their immunostimulatory potential in the MIMIC[®] system to determine assay dose range and analysis parameters. The second phase was a blinded study wherein 2 lots of each enoxaparin (branded and generic) was tested using the assay protocol defined in the pilot study. The third/follow-up phase of work was performed to confirm the results of the blinded study; this analysis included the Lot 1 and 2 complexes from the blinded study and a fresh batch of Lot 2 complexes prepared specifically for this phase of work. The heparin samples prepared and tested in each phase of study are shown in Table 1.

Reagents

Unfractionated heparin (average molecular weight of ~15 000 Da), branded LMWH enoxaparin (average molecular weight of ~4500 Da), and ULMWH (average molecular weight of ~2000 Da) were obtained from Sanofi. Sandoz and Amphastar enoxaparin syringes were purchased in the United States in 2012. Lot numbers for all heparinoids used are listed in Table 1.

Human native PF4 was obtained from washed and disrupted platelets by 2 rounds of heparin chromatography and gel-permeation chromatography as described previously.¹⁹ Although not shown here, all PF4 lots used in this study were highly purified, homogeneous (tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]), and mainly in the tetrameric form (tested by fast protein liquid chromatography [FPLC]).

Preparation of Peripheral Blood Mononuclear Cells

Apheresis blood products were collected from 47 donors (first phase: 15 donors; second phase: 20 donors; and third phase: 12

Table 2. Preparation of PF4–Heparin Complexes.

Heparin	Anti-Factor Xa Activity (IU/mg)	Final Concentration Heparin per 1 mg PF4 (µg/mL)	PF4–Heparin Ratio
UFH:	~180	150	6.66:1
ULMWH:	~253	245	4.08:1
Branded LMWH:	~110	245	4.08:1
US-Generic LMWH:	~110	245	4.08:1

Abbreviations: LMWH, low-molecular-weight heparin; PF4, platelet factor 4; UFH, unfractionated heparin; ULMWH, ultra low-molecular-weight heparin.

donors) at the OneBlood collection center (Orlando, Florida). (OneBlood is a not-for-profit blood center servicing regions of Florida, Alabama, and Georgia.) The study protocol and our donor program were reviewed and approved by Chesapeake Research Review Inc (Columbia, Maryland). All donors were in good health, and all blood products were negative for blood-borne pathogens as detected by standard blood bank assays.

Peripheral blood mononuclear cells (PBMCs) were enriched by Ficoll density gradient separation, according to standard laboratory procedures. After washing, PBMCs were cryopreserved in dimethyl sulfoxide-containing freezing media for extended storage in liquid nitrogen. Although effort is made to remove platelets from the PBMC preparations, a small and variable concentration of platelets does remain in the final product. Donor PBMCs were chosen at random from our pool for inclusion in this study.

Peripheral Blood Mononuclear Cells Assay

Peripheral blood mononuclear cells were prepared and cultured in serum-free X-VIVO 15 media (Life Technologies, Grand Island, New York) with UFH, branded enoxaparin (Lot number EEA1412E), and the US-generic enoxaparins produced by Sandoz (Lot number 917499) and Amphastar (Lot number EP002B2) at a dose of 10 µg/mL for 1 or 5 days. Platelet factor 4 alone (5 and 10 µg/mL) was used as the negative control. As a positive control, 50 ng/mL of the toll-like receptor (TLR) 4 agonist, lipopolysaccharide (LPS; *Escherichia coli* 0111: B4 lipopolysaccharide, Sigma-Aldrich, St Louis, Missouri), and 10 µg/mL of the TLR7/8 agonist, R848 (Invivogen, San Diego, California), were added to the constructs. The cells were harvested, washed, and labeled for viability with LIVE/DEAD Aqua (Invitrogen, Eugene, Oregon). The cells were then labeled with a multicolor antibody panel specific for cluster of differentiation (CD) 14, human leukocyte antigen-DR, antigen-presenting cell (APC) activation/maturation markers (CD86 and CD83), and lymphocyte markers (CD3 and CD19). All antibodies were purchased from eBiosciences (San Diego, California) or BD/Biosciences (San Jose, California). Data were acquired on a BD FORTESSA II flow cytometer (BD/Biosciences) and analyzed using FlowJo software (TreeStar Inc, Ashland, Oregon).

Preparation of PF4–Heparin Complexes

Platelet factor 4–heparin complexes were prepared using published protocols, with slight modification, to produce stoichiometric complexes at ~27 IU of heparin per mg of PF4.¹⁹ (Of note, PF4 complexes prepared with ULMWH contained ~62 IU ULMWH per mg PF4.) To achieve these stoichiometric ratios, highly purified native human PF4 (5.81 mg/mL in 0.75 mol/L salt buffer) was mixed with heparins and brought to a final concentration of 1 mg/mL in 0.15 mol/L salt buffer as shown in Table 2.

MIMIC[®] Peripheral Tissue Equivalent Assay

The peripheral tissue equivalent (PTE) construct of the MIMIC[®] system is designed to replicate the early processes of innate immunity (cytokines and APC activation/maturation) in response to test agents.^{16,20} The automated MIMIC[®] PTE module used in this study was built around our published manual technique.²⁰ Briefly, endothelial cells were grown to confluency atop a collagen matrix (PureCol; Advanced Biomatrix, San Diego, California). Thereafter, donor PBMCs were prepared from frozen stocks and applied to MIMIC[®] PTE assay wells. After a 90-minute incubation period, nonmigrated cells were washed away, leaving a small residual population of contaminating lymphocyte and platelets (although the amount of contaminants varies slightly from donor to donor, this controlled variable did not impact the comparative LMWH analysis because all of the test samples were evaluated against each other at the same time for a particular donor and experiment). Test agents, including heparins alone or PF4–heparin complexes, were then added to the constructs at concentrations indicated in the Results section. As a positive control, 50 ng/mL LPS and 10 µg/mL R848 were added to the constructs. The reverse-transmigrated cells were harvested after a 48-hour incubation period for cell surface marker phenotype (flow cytometry) using the protocol described under the “PBMC Assay” section. Culture supernatants of MIMIC[®] PTE assays were also analyzed for TFPI production via a commercial DuoSet enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc, Minneapolis, Minnesota) according to the manufacturer’s instructions. The ELISA plate was read on a Bio-Tek Synergy HT multiwell reader and data were analyzed using KC4 software (Bio-Tek Instruments, Winooski, Vermont). All reagents, cells, and media employed in the MIMIC[®] PTE readout were tested and certified as endotoxin free.

Enzyme-Linked Immunosorbent Assay for PF4 Detection

The ZYMUTEST PF4 assay is a sandwich ELISA designed with affinity-purified rabbit polyclonal antibodies specific for human PF4 (HYPHEN BioMed, Neuville-sur-Oise, France). The procedure was conducted according to manufacturer’s instructions (HYPHEN BioMed) to evaluate PF4 content in the PF4–heparin complexes prepared for this study. In brief, the PF4–heparin complexes (1 mg/mL) were diluted 1:1000 for a

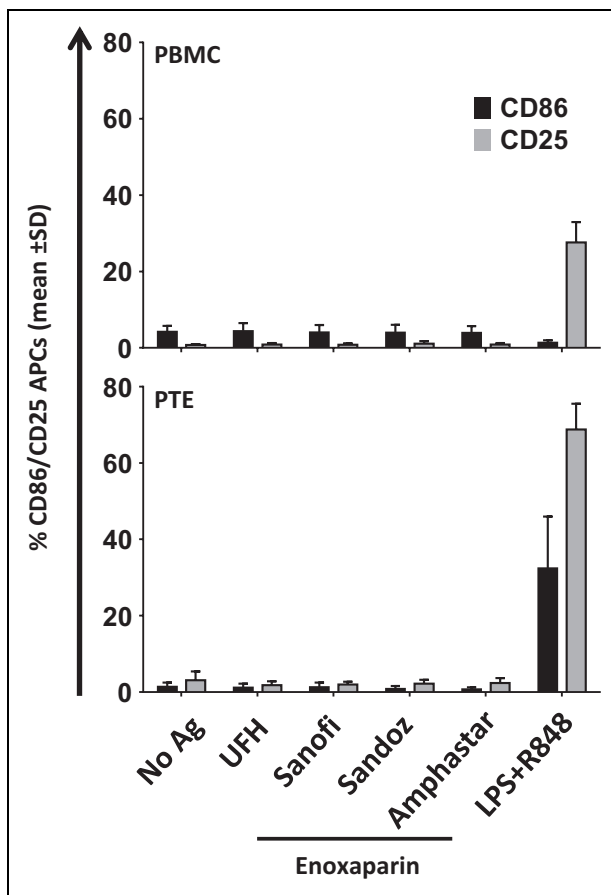


Figure 1. Heparins alone are not immunogenic in assay cultures. MIMIC[®] PTE (lower graph) and total PBMC (upper graph) cultures were treated with 10 μ g/mL heparins (Lot 1) or LPS/R848 for 48 hours and 24 hours, respectively. Thereafter, the cells were harvested and evaluated for their expression of CD86, CD25, CD14, and HLA-DR by flow cytometry. The plotted values represent the mean \pm SD for 10 donors; each experiment was repeated twice. Ag indicates antigen; APC, antigen-presenting cell; MIMIC-PTE, Modular IMMune In vitro Construct system-peripheral tissue equivalent; PBMC, peripheral blood mononuclear cells; SD, standard deviation; LPS+R848, 100 ng/mL of TLR4 agonist (LPS): lipopolysaccharide plus 10 μ g/mL of the TLR7/8 agonist R848.

PF4 concentration of 1 μ g/mL and then further diluted 1:100 to be within the assay dynamic range (0-10 ng/mL). From here, the assay was performed using standard immunosorbent techniques, except that the evaluation was done in 1.0 mol/L NaCl to recover a maximal amount of PF4 from PF4-heparin complexes. The assay plates were read at 450 nm.

Native Polyacrylamide Gel Electrophoresis Analyses

Platelet factor 4-heparin complexes were quantified for protein content (concentration) by Bradford assay. Thereafter, 25 ng of the complexes were separated by 4% to 20% gradient native polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions and visualized by silver staining (ProteoSilver Silver Stain Kit, Sigma-Aldrich) against molecular weight

standards. Pictures were taken with the Kodak GL 1500 imaging system (Kodak, Rochester, New York).

Size Exclusion Fast Performance Liquid Chromatography Analysis

Size exclusion fast performance liquid chromatography analysis of PF4 and PF4-heparin complexes was performed using a TSK 3000 SW column (30 cm height, 7.5 mm diameter, particle size 10 μ m) from TOSO HAAS (Stuttgart, Germany) and an Amersham-Pharmacia FPLC system (GE Healthcare, Pittsburgh, Pennsylvania). Analyses were performed in 0.15 or 0.75 mol/L NaCl in a 1% glycine buffer at pH 7.5.

Statistical Analysis

All statistical analyses and graphics were prepared using GraphPad InStat version 4.00 (GraphPad Software Inc, San Diego, California). One-way analysis of variance (ANOVA) and Bonferroni posttest analyses were employed to determine statistical significance; *P* values < .05 were considered statistically significant.

Results

Assessment of Immunostimulatory Potential of Noncomplexed Heparins in PBMC and MIMIC[®] PTE Assays

The purpose of this report was to evaluate the immunostimulatory potential of branded and generic enoxaparins in an in vitro model of the human innate immune system, termed the MIMIC[®] PTE construct. As published reports using human PBMCs and animal models suggest heparins in a noncomplexed state are not immunostimulatory,^{10,12,21-23} our first objective in this study was to confirm these observations in the MIMIC[®] PTE construct. In a 10-donor experiment, no changes in expression of 2 APC T-cell stimulatory ligands, CD86 and CD25, were detected in the MIMIC[®] based assay following treatment with branded or US-generic enoxaparins (Figure 1B). In contrast, both markers were strongly upregulated on APCs cultured in the presence of LPS and R848 (positive control) in the same experiment (Figure 1B). An analysis of total PBMCs, which serves as a standard of in vitro human immune evaluations and was included in this experiment to permit the benchmarking of the MIMIC[®] PTE results against published literature, generated similar results following LMWH treatment (Figure 1A). Of note, the positive TLR agonist control induced stronger upregulation of CD25 than CD86 in this assay (Figure 1A). To verify the observations shown in Figure 1 were not an artifact of the 24-hour PBMC and 48-hour MIMIC[®] PTE assay incubation periods being too short to generate APC phenotype changes, the PBMC assay was extended to 5 days but again showed no obvious effect of the heparins (data not shown).

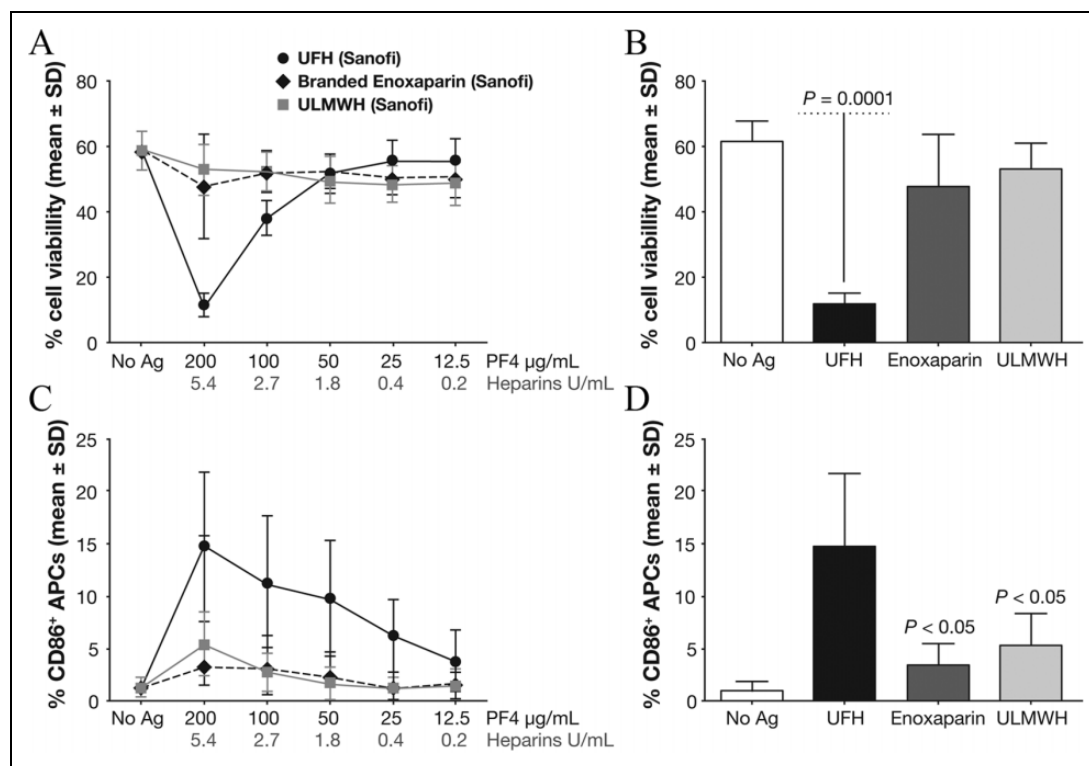


Figure 2. Demonstration of differential immune responses induced against PF4 complexes with UFH, LMWH (branded enoxaparin), and ULMWH in MIMIC[®] PTE assays. MIMIC[®] PTE assays were treated with a broad range of PF4–heparin complexes as indicated in the figure labels. After 48 hours, the reverse-transmigrated APCs were harvested and labeled with markers specific for a variety of parameters by flow cytometry. A, Cell viability was measured by LIVE/DEAD Aqua staining over the entire dose range and (B) statistical analysis of the viability data was calculated at the highest treatment dose. C, APC activation was measured by CD86 upregulation on the CD14⁺HLA-DR⁺ DC population; (D) a statistical assessment of CD86 expression is shown for the highest treatment dose. The plotted values represent the mean \pm SD for 14 donors studied in 5 distinct experiments. Ag indicates antigen; APC, antigen-presenting cell; DC, dendritic cell; LMWH, low-molecular-weight heparin; MIMIC[®] PTE, Modular Immune In vitro Construct system–peripheral tissue equivalent; PF4, platelet factor 4; SD, standard deviation; UFH, unfractionated heparin; ULMWH, ultra-low-molecular-weight heparin.

Complexes of Heparins With PF4 Are More Immunostimulatory Than Heparins Alone (Pilot Study)

Given that complexes of heparins and positively charged proteins, such as PF4, are thought to be more immunostimulatory than heparins alone,^{12,24} we anticipated the evaluation of heparin innate immune system engagement might be better achieved by comparing variant PF4–heparin complexes in the MIMIC[®] PTE construct. Therefore, in the pilot study (see Materials and Methods section and Table 1 for additional details), graded doses of PF4–heparin complexes (200 to 12.5 μ g/mL PF4 at a constant ratio to heparin) formed with UFH, branded enoxaparin, and ULMWH were examined for their impact on APC viability and activation in the MIMIC[®] PTE construct. (Both UFH and ULMWH were particularly crucial for this proof-of-concept study, since they were expected to trigger strong and weak immune responses, respectively, based on their anticipated capacities to form complexes with PF4.) As shown in Figure 2A, PF4–heparin complexes prepared with UFH triggered a steep reduction in cell numbers in a dose-dependent manner; for example, at the highest concentration, cell viability was decreased to 18.65% \pm 14.5% from 61.6%

\pm 6.6% in the no-complex control (ANOVA, $P \leq .0001$; Figure 2B). In contrast, even at the highest treatment dose, PF4–ULMWH complexes failed to trigger a statistically significant decrease in cell numbers (55.06% \pm 7.2%; ANOVA, $P > .05$). Although they were not completely inert in these experiments, PF4–branded enoxaparin triggered only minimal (not statistically significant) reductions in cell viability (Figure 2A and B).

The evaluation of APC activation in the MIMIC[®] PTE construct, as measured by the heightened expression of CD86 on the harvested DC population, yielded data trends similar to those described in Figure 2A and B. The PF4–UFH complexes were the most immunostimulatory, inducing strong and statistically significant CD86 upregulation at all but the lowest treatment dose (Figure 2C and D). The PF4–branded enoxaparin and PF4–ULMWH complexes, on the other hand, triggered only minimal increases in APC activation even at the highest treatment dose (3.2% \pm 1.9% and 5.4% \pm 2.9%, respectively). Taken together, these pilot study results indicate (1) the MIMIC[®] PTE system can be used to examine the immunostimulatory potential of protein-complexed heparinoids, (2) only protein-complexed heparins, but not heparins alone, are immunogenic in this system, and (3) large and small molecular

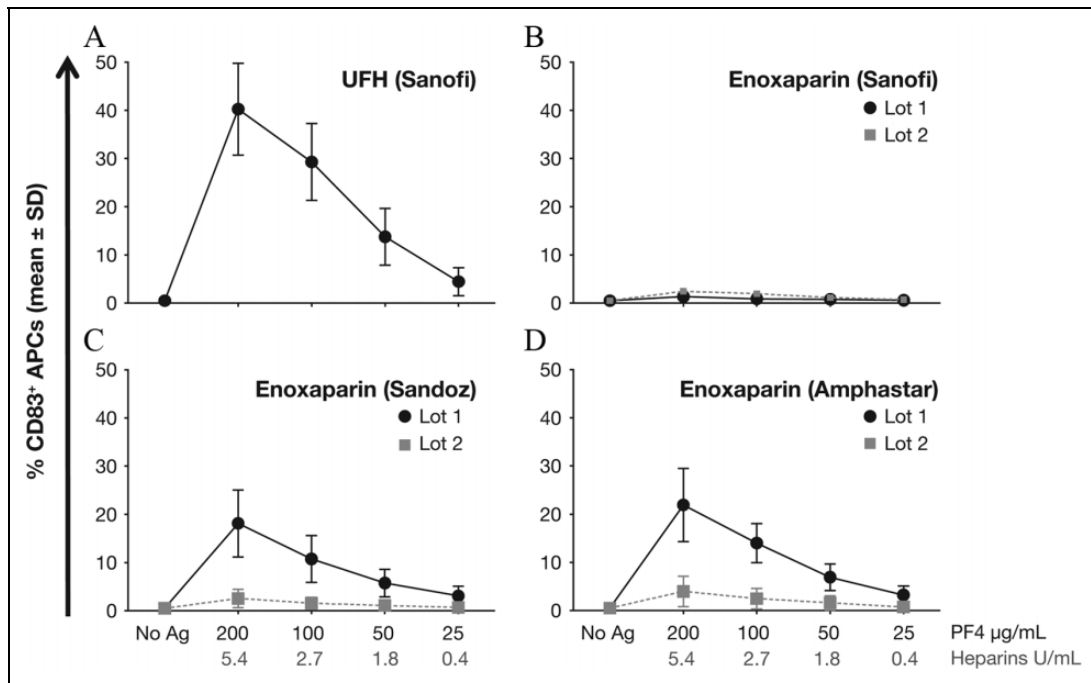


Figure 3. Branded and generic enoxaparins have different capacities to activate APCs in the MIMIC[®] PTE construct. MIMIC[®] PTE assays were treated for 48 hours with the indicated concentrations of PF4 complexes prepared with (A) UFH, (B) Sanofi enoxaparin, (C) Sandoz enoxaparin, or (D) Amphastar enoxaparin. Thereafter, the cells were harvested and examined for CD83 expression on the CD14⁺HLA-DR⁺ DC population by multiparameter flow cytometry. The data plotted show the means \pm SD for 20 donors examined in 5 distinct-blinded experiments. Ag indicates antigen; APC, antigen-presenting cell; DC, dendritic cell; MIMIC[®] PTE, Modular Immune In vitro Construct system-peripheral tissue equivalent; PF4, platelet factor 4; SD, standard deviation; UFH, unfractionated heparin.

weight products have differential capacities to modulate immune function in the MIMIC[®] PTE.

Branded and Generic Enoxaparins Demonstrated Differential Immunostimulatory Potentials (Blinded Study)

Having demonstrated the capacity of the MIMIC[®] PTE system to support differential responses against variant heparinoids in the pilot study, we next examined the immunostimulatory potential of PF4 complexes prepared with UFH and 2 lots each of branded (Sanofi) and generic (Sandoz and Amphastar) enoxaparins in the MIMIC[®] PTE system in a blinded fashion (Blinded Study; Table 1). (The complexes used in this study were prepared at the same time using a new single batch of PF4.) In this analysis, a stringent marker of APC maturation, CD83,^{20,25} was used as the primary readout of activated MIMIC[®] PTE-derived APCs.

Consistent with the pilot study results (Figure 2), PF4–UFH complexes prepared for this phase of work triggered a significant dose-dependent reduction in cell numbers (data not shown). Likewise, PF4–UFH complexes induced high levels of APC activation in a dose-dependent manner; Figure 3A shows the peak response was $40.2\% \pm 9.5\%$; ANOVA, $P \leq .0001$ at the highest treatment dose. Regarding the evaluation of variant enoxaparins, no reduction in cell viability was observed when MIMIC[®] PTE cultures were treated with PF4 complexes prepared with either the branded or the generic enoxaparins

(data not shown). However, although the APC activation profile for PF4–branded enoxaparin was consistently low across the 2 test lots, innate response profiles triggered by PF4 complexes prepared with generic enoxaparins (Sandoz and Amphastar) were significantly variable between the 2 lots of each product (Figure 3B, C, and D; ANOVA, $P \leq .0001$ at the highest treatment dose). Also, statistical analyses revealed that the PF4–enoxaparin complexes prepared with Lot 1 Sandoz and Amphastar enoxaparins generated significantly different innate responses than complexes prepared with either lot of the branded product (ANOVA, $P \leq .0001$ at the highest treatment dose). See Table 3 for all statistical comparisons generated for this data set.

Secretion of TFPI Differentiated Branded and Generic Enoxaparin Function in MIMIC[®] PTE Assays (Blinded Study)

Tissue factor pathway inhibitor plays a crucial role in regulating the coagulation cascade and is one of the critical pharmacodynamic markers of heparin function.^{18,26} As TFPI is constitutively synthesized by vascular endothelial cells,²⁷ and endothelial cells serve as a principal component of the MIMIC[®] PTE construct, we questioned whether it could be used as a marker of heparin function in this study. As a proof-of-concept experiment, cultures harvested from MIMIC[®] PTE assays treated with noncomplexed UFH and variant enoxaparins showed 2- to 3-fold increases in free TFPI

Table 3. Statistical Analysis (1-Way ANOVA With Bonferroni Posttest) of CD83 Upregulation in APCs Stimulated With PF4–Enoxaparin Complexes Containing Branded and US-Generic Enoxaparins That Were Prepared for the Blinded Study.

		Branded Enoxaparin (Lovenox)		Generic Enoxaparin (Sandoz)		Generic Enoxaparin (Amphastar)	
		Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
Branded Enoxaparin (Lovenox)	Lot 1		NS	$P < .0001$	NS	$P < .0001$	NS
	Lot 2			$P < .0001$	NS	$P < .0001$	NS
Generic Enoxaparin (Sandoz)	Lot 1				$P < .0001$	$P < .05$	$P < .0001$
	Lot 2					$P < .0001$	NS
Generic Enoxaparin (Amphastar)	Lot 1						$P < .0001$
	Lot 2						

Abbreviations: ANOVA, analysis of variance; APC, antigen-presenting cell; NS, not statistically significant; PF4, platelet factor 4.

accumulation over the baseline control condition (Figure 4A). Subsequently, the effect of PF4–heparin complexes on TFPI production in MIMIC[®] PTE assays was examined using the same culture supernatants profiled in Figure 3. When compared against the phenotype results, it is notable that TFPI production was inversely correlated with APC activation, such that strongly immunostimulatory complexes triggered significant decreases in TFPI production below the baseline level observed in control condition. For example, the highly immunostimulatory PF4–UFH complexes reduced TFPI production by approximately 50% and both lots of PF4–branded enoxaparin complexes, which were only weakly immunostimulatory, increased TFPI production ~3- to 4-fold above the baseline level (Figure 4B). On the other hand, complexes formed with the generic enoxaparins (Sandoz and Amphastar) generated variable TFPI responses (Figure 4B) that were inversely matched to the APC phenotype data shown in Figure 3.

Confirmation of Blinded Study Results (Follow-Up Study)

An additional study was performed using a new batch of PF4–enoxaparin complexes (prepared with Lot 2 of branded and generic enoxaparins) to ensure the blinded study observations were not an assay artifact related to the batch of PF4 and/or the manufacture of the PF4–heparin complexes used in that study. As well, for secondary confirmation of the blinded study results, the PF4–enoxaparin complexes evaluated in those experiments were included in this phase of work (Table 1). Of note, although our preference would have been to retest new batches of compounds from both lots of branded and US-generic enoxaparins, we only had sufficient material remaining from Lot 2 to prepare new batches of complexes for this phase of work.

The follow-up study reproduced what was found in the blinded study: (1) PF4–heparin complexes prepared for the blinded study gave identical results in the new 10-donor study group, (2) fresh complexes prepared with a new batch of PF4 and the Lot 2

branded and generic enoxaparins generated the same results as those obtained in the blinded study, and (3) the strong negative correlation between APC activation and TFPI production in the blinded study was confirmed in this set of experiments. As can be seen in the graphs in Figure 5A and B, the 3 batches of PF4–branded enoxaparin, prepared using 2 different enoxaparin lots, were highly consistent in terms of immunostimulatory profiles and capacity to augment TFPI production. Conversely, there were clear differences between the 2 US-generic enoxaparin product lots regarding their capacity to trigger immune cell activation and modulate TFPI production (Figure 5A and B). The results of the blinded and follow-up studies were integrated together into Table 4 to show the differential response profiles of PF4 complexes prepared with branded and US-generic enoxaparins.

Platelet Factor 4 Complexes of Generic and Branded Enoxaparins Generated Distinct Analytical Profiles

We considered the possibility that the differential immunostimulatory potential of PF4 complexes prepared with branded and US-generic enoxaparins may have resulted from possible differences in the physicochemical properties of these macromolecules. To test this theory, the size and strength of intramolecular interactions of blinded study (Lot 1, Batch 1) complexes were tested via SE-FPLC analysis under physiologic (0.15 mol/L) and high (0.75 mol/L) NaCl concentrations. Human PF4 without heparin, which was used to establish a baseline reading in the chromatography assay, generated 2 peaks (principally monomers and dimers) that eluted at graph positions 11 to 13 under physiologic (0.15 mol/L) NaCl conditions (Figure 6A, upper row). At a high (0.75 mol/L) salt concentration, PF4 was shown to be homogeneous and in the tetrameric form, that is, an elution peak at position ~10 on the graph (Figure 6A, bottom row). This result is consistent with prior studies showing that a high salt concentration favors the formation of tetrameric PF4 molecules.¹³ When PF4 was complexed with branded

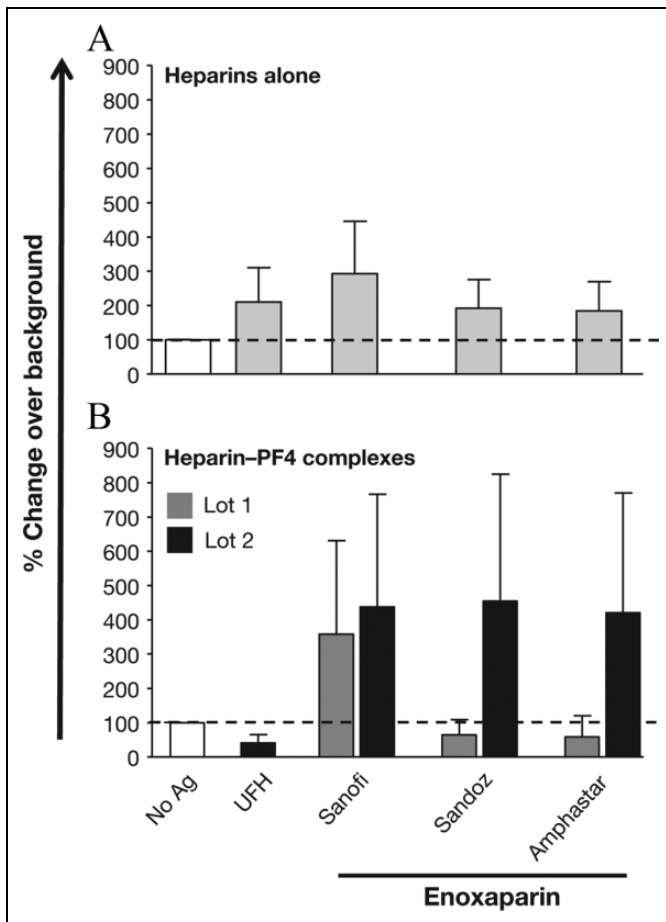


Figure 4. Brand and generic enoxaparins have different capacities to induce TFPI secretion in the MIMIC[®] PTE construct. A, MIMIC[®] PTE cultures were treated with heparins alone (Lot 1) for 48 hours, the culture supernatants were then harvested and examined for TFPI secretion by ELISA. The data show the means \pm SD of the percentage change over the control (no treatment condition) for 5 donors. B, Culture supernatants from the blinded study cultures shown in this figure and in Figure 3 were harvested at 48 hours posttreatment and examined for TFPI by ELISA. The data show the means \pm SD of the percentage change over the control (no treatment condition) for 10 donors included in the blinded study. Ag indicates antigen; ELISA, enzyme-linked immunosorbent assay; MIMIC[®] PTE, Modular Immune In vitro Construct system-peripheral tissue equivalent; PF4, platelet factor 4; SD, standard deviation; TFPI, tissue factor pathway inhibitor; UFH, unfractionated heparin.

enoxaparin, it generated a single peak that eluted at graph position \sim 6 under physiologic salt concentrations (Figure 6A, upper row), which suggests the branded product forms homogeneous complexes smaller than the 500-kDa molecular weight cutoff of the column. These complexes also appeared to readily dissociate from the polysaccharides in the high NaCl concentration buffer, yielding a major peak of tetrameric PF4 that eluted at position \sim 10 on the graph (Figure 6A, bottom row). The US-generic enoxaparins yielded quite a distinct SE-FPLC profile. First, the complexes failed to generate peaks under physiologic salt conditions, which suggest the complexes were larger than the 500-kDa molecular weight cutoff of the column

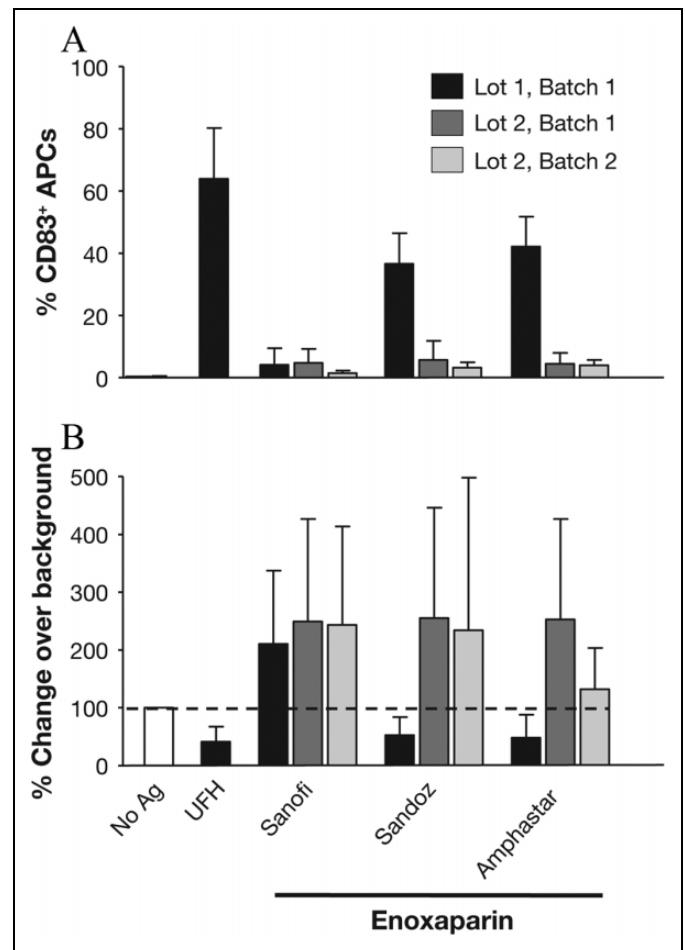


Figure 5. TFPI production is inversely correlated with APC activation in MIMIC[®] PTE assays. MIMIC[®] PTE assays were treated with PF4 complexes prepared with variant heparinoids. A, APC activation, as measured by CD83 expression (CD14⁺HLA-DR⁺ DCs), is shown graphically for all samples included in the assay. B, TFPI secretion, represented as a percentage-change compared with the no-treatment condition, is displayed for the indicated treatment conditions. The data show the mean \pm SD for 10 donors examined in 4 distinct experiments. Ag indicates antigen; APC, antigen-presenting cells; DCs, dendritic cells; MIMIC[®] PTE, Modular Immune In vitro Construct system-peripheral tissue equivalent; PF4, platelet factor 4; SD, standard deviation; TFPI, tissue factor pathway inhibitor; UFH, unfractionated heparin.

(Figure 6A, upper row). Second, these complexes appeared to poorly dissociate under high salt concentrations, since there were only minor PF4 peaks at the graph elution position of \sim 10 in the Amphastar and Sandoz conditions (Figure 6A, bottom row).

We also analyzed the Lot 1, Batch 1 complexes by nonreducing native PAGE as a secondary method to examine the size of the macromolecules contained in these preparations. Referring to the image of the 4% to 20% gradient gel shown in Figure 6B, it is evident all of the complex preparations generated a band in the 150 to 250 kDa range, but the band was substantially more intense in the branded enoxaparin condition than with either of the US-generic products. Similar to the conclusions drawn with

Table 4. Summary of Results.^a

	PF4 Complexes Prepared With								
	Branded Enoxaparin (Sanofi)			US Generic Enoxaparin (Sandoz)			US Generic Enoxaparin (Amphastar)		
	Lot 1 (EEA1412E)	Lot 2 (EEB1402)		Lot 1 (917499)	Lot 2 (917999)		Lot 1 (EP002B2)	Lot 2 (EP099J1)	
	Batch 1	Batch 1	Batch 2	Batch 1	Batch 1	Batch 2	Batch 1	Batch 1	Batch 2
APC Activation:	NC	NC	NC	↑	NC	NC	↑	NC	NC
TFPI Production:	↑	↑	↑	↓	↑	↑	↓	↑	↑

Abbreviations: APC, antigen-presenting cell; TFPI, tissue factor pathway inhibitor.

^a Key: NC, no change; ↑, increased; ↓, reduced.

the SE-FPLC analysis (Figure 6A), we suspect the US-generic enoxaparins generated lower intensity bands because the complexes were too large to enter into the 4% to 20% gradient gel matrix. To rule out the alternative possibility that the US-generic enoxaparin preparations simply contained less material, we used the ZYMUTEST PF4 ELISA methodology (see the Materials and Methods section for additional details) to show each of the PF4–heparin complex preparations contained nearly equal amounts of PF4 (data not shown).

Given that the MIMIC[®] PTE evaluations showed the 2 lots of US-generic enoxaparins had differential capacities to trigger immune activation and modulate TFPI production, we next evaluated whether the 2 lots of US-generic products would also generate unique SE-FPLC profiles under the dissociating high salt condition. As can be seen in Figure 7, the SE-FPLC profile of the complexes prepared with Lot 1, Batch 1 Amphastar generated a minor peak at the elution position of 10 on the graph (similar to Figure 6A, bottom row), whereas Lot 2, Batch 1 Amphastar generated a major peak at the graph elution position of 10. These SE-FPLC observations suggest the 2 lots of Amphastar generated PF4 complexes with unique physicochemical profiles; coupled with the results of Figure 5, it appears tightly associated complexes (yielding smaller PF4 peaks) are more immunostimulatory than more easily dissociated complexes that are capable of generating larger peaks at elution point 10.

Discussion

In this study, we evaluated the immunostimulatory potential of PF4–heparin complexes in an in vitro model of human innate immunity, the MIMIC[®] PTE system. Our results demonstrating protein–heparin complexes (and not heparins alone) are immunostimulatory in the MIMIC[®] PTE construct are consistent with published results showing PF4–UFH complexes, but not UFH alone, can trigger interleukin 12 production by murine bone marrow-derived APC.¹² Also, the comparative analysis of heparins of different molecular weights (UFH, LMWH, and ULMWH) in the pilot study of this report provides additional

evidence that PF4–heparin macromolecules drive immune reactions because only PF4–UFH complexes were capable of triggering the activation of the MIMIC[®] PTE construct. The UFH has a strong propensity to complex with proteins,²⁸ whereas ULMWH and LMWHs are generally thought to have lesser capacities to form protein complexes.⁹

Based on the promising pilot study results suggesting an in vitro platform of human immunity could be used to examine heparin immunostimulatory potential, we subsequently used the system to show PF4 complexes prepared with US-generic and branded enoxaparins can trigger differential innate immune response and TFPI secretion profiles in the MIMIC[®] PTE construct. Our study is not the first to suggest branded and generic enoxaparins can have unique biological/functional properties. For example, Walenga et al²⁹ reported pharmacological differences (inhibition of clot formation and TFPI secretion) between branded (Lovenox) and generic (Sandoz) enoxaparin. However, a novel finding of this study is that the immunostimulatory potential of the generic enoxaparins varied considerably between the 2 lots of material included in the study. Although it is possible the manufacture process of LMWHs might impart the final products with unique capacities to engage the immune system through protein complex formation, it should be noted that additional lots of material would need to be evaluated to further address this issue.

Through multiple evaluations of distinct lots and batches of PF4–heparin complexes, we found a strong (inverse) correlation between immune activation and TFPI secretion in the MIMIC[®] PTE construct. Considering the possibility of a causal relationship between these 2 assay readouts, we postulate large PF4–heparin complexes trigger inflammatory responses that actively suppress TFPI production by endothelial cells in the MIMIC[®] PTE construct. In support of this hypothesis, we found LPS triggered reduced TFPI production in the MIMIC[®] PTE construct (data not shown) and a published study showed the same inflammatory agent failed to induce TFPI secretion in the endothelial cells.³⁰ Alternatively, we hypothesize PF4–heparin complexes with low inflammatory

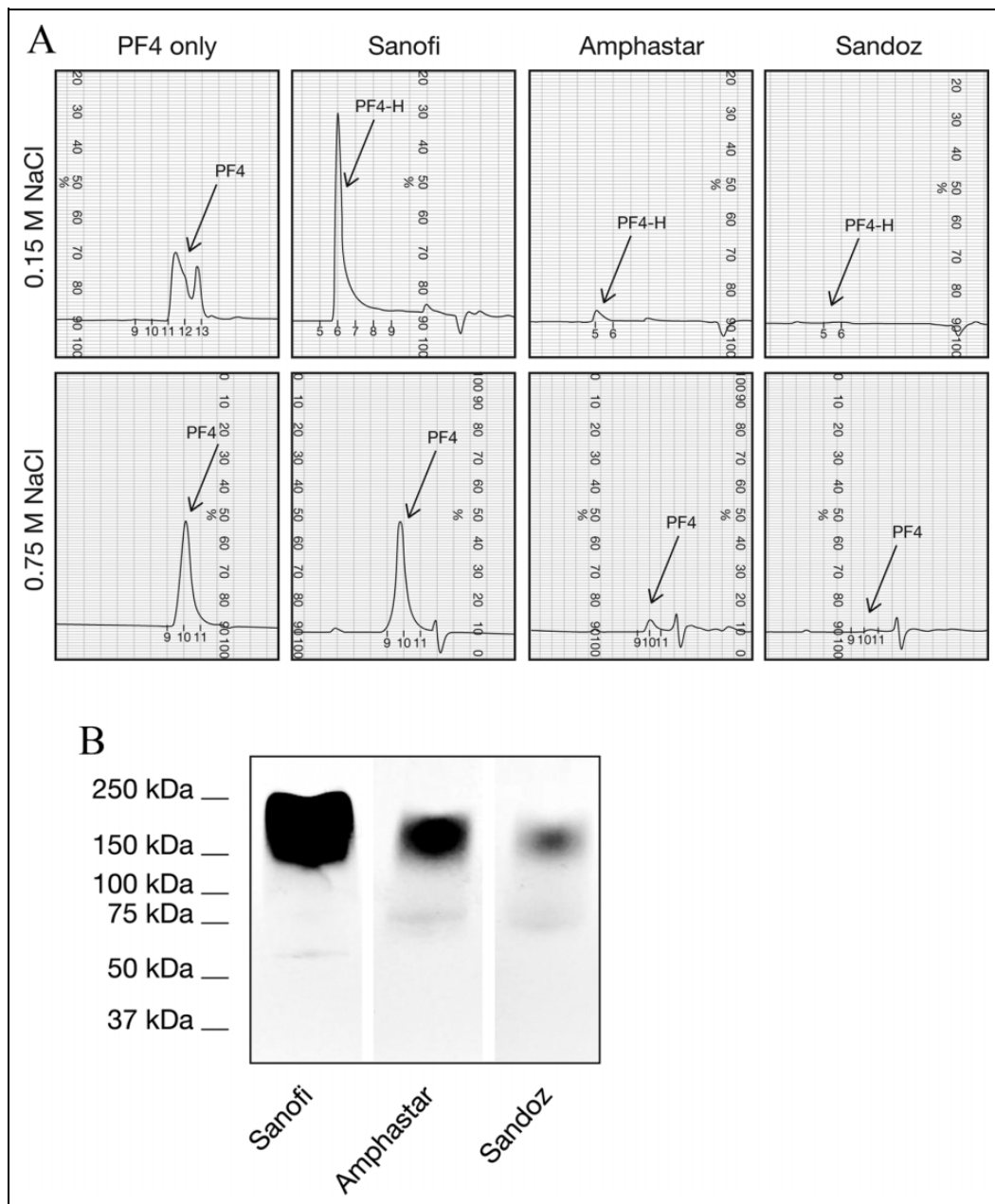


Figure 6. Demonstration of differential release of PF4 from complexes formed with branded and US-generic enoxaparins. **A**, Chromatogram of SE-FPLC analysis of PF4 alone and all Lot 1, Batch 1 PF4–enoxaparin complexes performed at 0.15 mol/L NaCl (upper panel) and 0.75 mol/L NaCl (lower panel). **B**, 4% to 20% gradient native PAGE gel analysis of PF4–enoxaparin complexes under nonreducing conditions followed by silver staining to resolve the protein bands. These data are representative of 3 independent analyses. ELISA indicates enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; PF4, platelet factor 4; PF4–H, PF4–heparin complex; SD, standard deviation; SE-FPLC, size exclusion-fast performance liquid chromatography.

potential are structured in such a manner that the PF4 is capable of engaging the endothelial cells to produce TFPI, whereas PF4 in highly immunostimulatory complexes is structured in such a manner that precludes it from engaging endothelial cells to secrete TFPI. Regardless of the potential mechanism, these results are well in line with clinical studies suggesting TFPI has strong anti-inflammatory properties.³¹

In concordance with the differential immunostimulatory and TFPI data reported here for the US-generic enoxaparins, we

observed marked differences in the size and molecular interactions of complexes formed between PF4 and the branded and US-generic enoxaparins via SE-FPLC and native PAGE gel analyses. Specifically, the results of these studies suggest branded enoxaparins can form complexes with PF4, but complexes formed between PF4 and the Lot 1 US-generic enoxaparins were larger and more tightly associated (less susceptible to salt concentrations that promote molecular dissociation). These differences, which correlated quite well with

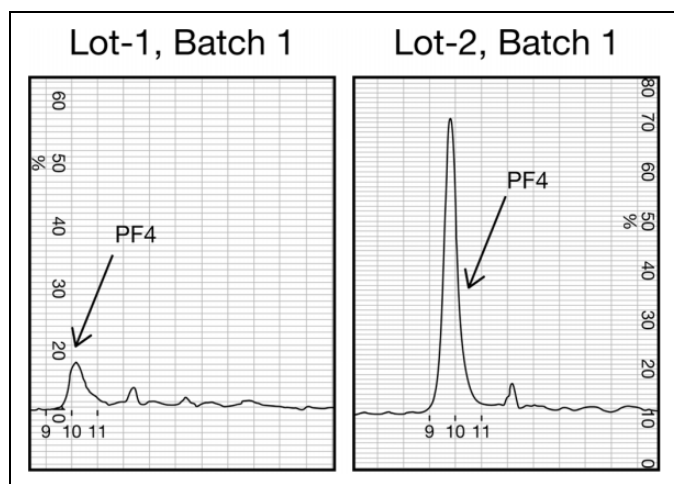


Figure 7. Differential SE-FPLC profiles of PF4–enoxaparin complexes prepared with 2 distinct lots of Amphastar. SE-FPLC analysis of Lot 1, Batch 1 and Lot 2, and Batch 1 Amphastar complexes was performed at 0.75 mol/L NaCl. PF4, platelet factor 4; SE-FPLC, size exclusion-fast performance liquid chromatography.

the immune activation and TFPI results generated in the MIMIC[®] PTE system, could potentially be related to the raw material (UFH) used to produce the LMWHs or to the depolymerization process used to generate the final product that, in some cases, can generate highly sulfated oligosaccharides or others glycosaminoglycans capable of forming ultra-large, potentially activating PF4–heparin complexes.^{9,13,32,33} Given the complexity of the manufacturing processes used to derive these products from biological samples, it is possible any of these factors might contribute to the differences observed in the results presented here.

In conclusion, we have used an in vitro model of human immunity to examine the innate immunostimulatory potential of heparinoids. The level of immunostimulation triggered by PF4–heparin complexes was found to be variable based on the form of heparin tested: UFH was the most stimulatory; branded enoxaparin and ULMWH were consistently nonstimulatory; and the generic versions, Sandoz, and Amphastar, were variably stimulatory depending on the product lot used in the evaluation. The distinct immune signatures of the US-generic enoxaparins might reflect variations in the manufacturing process used to prepare the LMWH that could lead to differences in the capacities of the final product lots to form complexes with PF4 and other positively charged proteins. This conclusion is supported by the analytical data generated in this study, suggesting the highly immunostimulatory lots of US-generic enoxaparins formed ultra-large, stable complexes with PF4. Finally, our results showing that PF4-complexed heparins, but not heparins alone, can trigger immune activation lend support to the concept of using such complexes as a screening for immunostimulatory potential and LMWH lot assessment, although further studies are warranted to elucidate pathophysiological relevance of these in vitro findings.

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Authors' Note

The authors are fully responsible for all content and editorial decisions related to the development of the manuscript.

Declaration of Conflicting Interests

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