

Pentosan Polysulfate and Heparin Exhibit Comparable Interactions with Platelet Factor 4, Suggesting a Potential Risk of Thrombocytopenia

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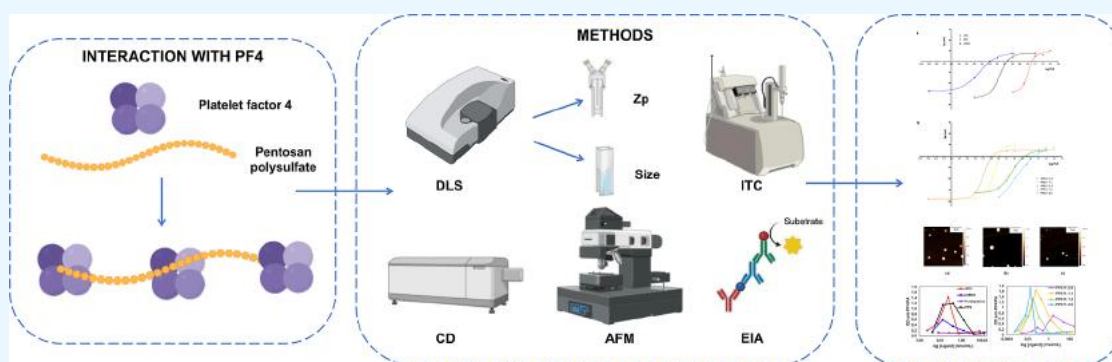
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ABSTRACT: Pentosan polysulfate (PPS) is an approved drug for the treatment of interstitial cystitis in humans and osteoarthritis in animals. This semisynthetic highly sulfated polysaccharide shares structural similarities with heparin and also interacts with platelet factor 4 (PF4), the key protein implicated in thrombocytopenia, a serious side effect of heparin administration. Thrombocytopenia arises from an immune response to structural features of multimeric complexes of heparin and PF4, although the prediction of disease progression in patients is complicated by the variable polyclonal and polyspecific response. The potential risk of provoking a similar response to PPS or materials derivatized with PPS, which could include subcutaneous or intravenous applications for other therapeutic goals, therefore needs to be assessed. In the absence of a clear proxy measurement for the risk of PPS to induce HIT, the ability of PPS and its fractions to interact with PF4 was examined from a broad structural perspective, employing orthogonal techniques, which were compared with unfractionated heparins (UFHs) and low-molecular-weight heparins (LMWHs). Zeta potential analysis, isothermal titration microcalorimetry, and circular dichroism showed that PPS interacts with PF4 in a manner dependent on its molecular weight, exhibiting behavior intermediate between that of LMWH and UFH. The interaction of PPS size-separated fractions with PF4 also exhibited a dependence on M_w ; higher M_w corresponding to stronger interactions, and the same trend was confirmed by atomic force microscopy. Interestingly, despite PPS forming complexes with PF4, and the complexes formed with PPS fractions being smaller than those formed with UFH and LMWH, enzyme immunoassay studies nevertheless demonstrated the formation of antigenic complexes. Since PPS provokes comparable interactions with PPS, the results suggest that close monitoring of potential thrombocytopenia effects will be necessary when considering PPS dosing, especially for intravenous applications.

1. INTRODUCTION

Pentosan polysulfate (PPS) is a highly sulfated polysaccharide of semisynthetic origin, obtained from the exhaustive *O*-sulfation of glucuronoxylan found in beechwood.¹ PPS has been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) and is marketed as Elmiron for the treatment of interstitial cystitis and bladder pain syndrome.^{2,3} PPS is composed of a heterogeneous mixture of polysaccharides, consisting of a backbone of repeating β (1–4)-linked D-xylose-2,3-*O*-disulfate units, with a low degree of branching through (1–2)-linked 2,3-di-*O*-

sulfated 4-*O*-methyl-D-glucuronic acid (MGA) residues.^{4,5} The structure is reported in [Figure S1](#). Owing to its sulfation, it has often been compared to heparin and, like heparin, PPS has been shown to possess anticoagulant activity,⁶ to demonstrate

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antiviral action against human immunodeficiency virus,⁷ SARS-CoV-2,^{8,9} and influenza,¹⁰ and to possess anti-inflammatory activities.¹¹ It has also been shown to inhibit hepcidin expression,¹² and its use has been evaluated for the treatment of mucopolysaccharidosis.^{13,14} The use of heparin as an anticoagulant can be limited, however, by an adverse immune reaction known as thrombocytopenia (heparin-induced thrombocytopenia (HIT)). This potentially serious condition arises when IgG antibodies recognize complexes formed between platelet factor 4 (PF4) and heparin. This polyclonal and polyspecific response occurs in particular when secondary structural changes are induced upon binding heparin to form tetrameric PF4 complexes and include increased proportions of antiparallel beta-strands, compared to their monomeric counterparts.¹⁵ The incidence of this antibody-mediated response varies considerably depending on the clinical scenario and the type of heparin administered, higher prevalence being observed in certain patient populations undergoing prolonged or intensive treatment.^{16,17}

Pentosan polysulfate is also sometimes employed as an anticoagulant in, for example, the treatment of venous thrombosis. Clinical manifestations resembling HIT have been reported.¹⁸ However, at the time most cases were described, antibody assays were not yet available; additionally, some patients received not only PPS but also heparin. Functional assays suggested an association of the clinical symptoms with PPS-induced thrombocytopenia.^{18–23} The current state of knowledge regarding those structural features that underly the ability of PPS to induce HIT is not sufficiently well understood to provide an explanation of the mechanism nor reveal which structural or biochemical characteristics could serve as proxy measures, or predictors, of its extent. Here, we assess how closely PPS resembles heparin in its interaction with PF4. In the absence of any defined proxy measure for the likelihood of including HIT, we therefore employed a broad, multifaceted, and unbiased approach, encompassing several complementary physicochemical and biochemical properties to compare the interactions between PPS and PF4 with those between unfractionated heparin and PF4.

Conventional HIT is diagnosed routinely with a screening assay for the detection of antibodies binding to PF4/heparin complexes. As binding of antibodies is rather unspecific, functional assays showing the potential of some of these antibodies to activate platelets *in vitro* using heparin-induced platelet activation assay (HIPA) or serotonin release assays (SRAs) are necessary, and only these activating antibodies are clinically relevant.²⁴ Monomeric PF4 consists (from N to C termini) of three antiparallel beta-strands and one α helix. Upon heparin binding, the tetrameric form is stabilized, exposing an immunogenic region that is variable, depending on the size of the bound heparin, or heparin-derived oligosaccharide.¹⁵ The proportion of beta-strand increases on complexation with heparin, and manifold antibodies, which are polyclonal and polyspecific, have been identified. Furthermore, not all of the antibodies are associated with immunological problems, and antibody formation does not directly correlate with the extent of immunogenicity in mice,¹⁷ although there is evidence that complexes with positive zeta potential (Zp) cause stronger immunological responses.²⁵

The nature of the interactions between heparin and PF4 is known to differ for unfractionated and low-molecular-weight heparin, and, in order to explore whether a similar relationship occurred in the case of PPS, it was fractionated with anionic

exchange chromatography as reported by Bertini et al.⁸ The interactions of its fractions, possessing varying molecular weights, and PF4 were also evaluated. Complex formation was analyzed using a combination of orthogonal analytical techniques including dynamic light scattering (DLS), zeta potential (Zp), atomic force microscopy (AFM), isothermal titration calorimetry (ITC), and circular dichroism (CD) spectroscopy. The size of the resulting macromolecular complexes depended strongly on the molar ratio of PF4 to the ligand; the largest aggregates formed at ratios corresponding to net charge neutrality. Additionally, an immunoassay was developed to evaluate the binding of a monoclonal antibody (clone KKO), which mimics the function of pathogenic human antibodies involved in immune responses associated with PF4/polyanion complexes.²⁶

If PPS appears to provoke comparable interactions with PF4 and is degraded less readily than heparin, this would suggest that close monitoring of potential thrombocytopenia effects will be necessary, as they are for heparin, when considering PPS dosing, especially for intravenous applications.

2. RESULTS AND DISCUSSION

2.1. Photocorrelation Spectroscopy

Literature reports²⁷ that certain polyanions can interact with the positively charged PF4, forming large and antigenic complexes. Photocorrelation spectroscopy can be used to measure and monitor the average radius of these PF4/ligand complexes. Generally, titration of PF4 with a negatively charged ligand shows an increase in particle size after the formation of nonspecific electrostatic binding between the ligands and the protein.²⁸ After reaching a maximum point, however, the average size may decrease due to potential saturation or aggregation effects. This leads to a typical “bell curve” shape, in which the peak of the curve corresponds to maximum aggregation. Aggregation studies by PCS on PPS exhibit this typical behavior, with a maximum aggregation achieved between PF4-ligand molar ratio (PLR) of 4 and 5 and with an average radius of about 540 nm (Figure 1a). PPS results were compared to UFH and LMWH data, which revealed that UFH/PF4 complexes reach peak aggregation at a higher PLR (between 12.8 and 16.0) and have an average radius exceeding 1000 nm, while LMWH/PF4 complexes display a maximum aggregation at PLR values between 3.2 and 2.0 and sizes consistently larger than PPS aggregates, but below 1000 nm.

The impact of PPS fractions with different molecular weights on complex formation was evaluated (Figure 1b). A decrease of fraction size corresponded to a reduction in the average size of the aggregates, as observed and reported in the literature for heparin fractions.²⁸ The behavior of the aggregates of PPS fractions, especially for those at low M_w , however, was characterized by flatter curves, making the identification of the point of maximum aggregation more difficult. Unlike the other PPS fractions, PPS Fraction 2.0, which had the highest M_w (17 kDa), showed maximum aggregation of a PLR between 4.0 and 6.4, with aggregates averaging 700 nm, similar to PF4/LMWH complexes (Table 1). At low M_w , the PPS fractions exhibited a PLR comparable to that of PF4/LMWH, but with a smaller average radius (Table 1).

2.2. Zeta Potential

The interaction between PF4 and ligands (PPS and heparins) is driven by electrostatic forces, and complex formation follows

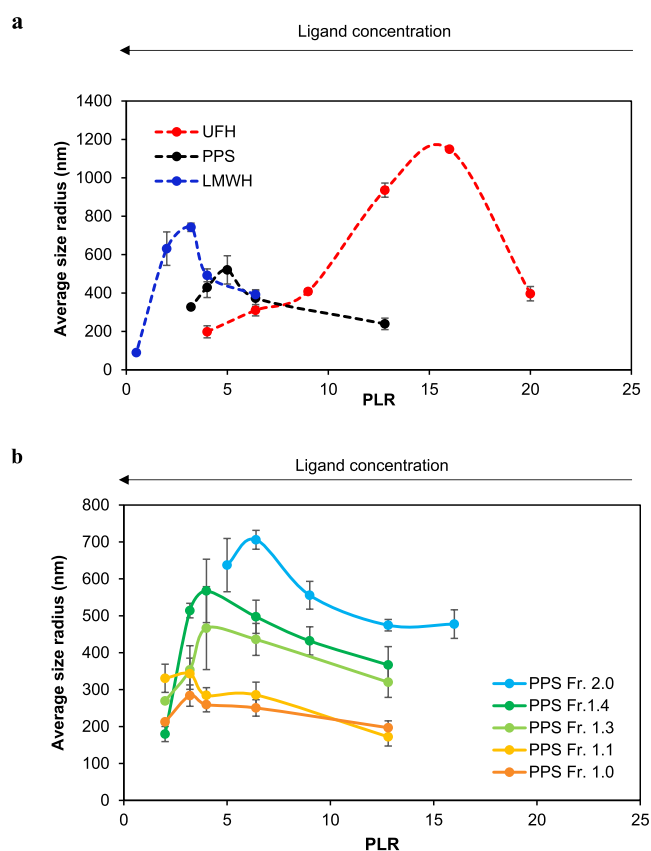


Figure 1. PCS measurements of PF4/heparin or PPS aggregates. Average size over the PLR of (a) PPS (black circle solid), LMWH (blue circle solid), and UFH (red circle solid). (b) PPS fractions: in detail are reported PPS Fr. 1.0 (orange circle solid), PPS Fr. 1.1 (yellow circle solid), PPS Fr. 1.3 (green circle solid), PPS Fr. 1.4 (teal green circle solid), and PPS Fr. 2.0 (cyan circle solid). The standard deviation is shown for every sample.

Table 1. Summary of PCS and Z_p Results of LMWH, UFH, PPS, and Its Fractions in Terms of Molecular Weight, Polydispersity Index (PDI), Protein–Ligand Complexes, Average Radius, and PLR at the Point of Neutral Zeta Potential^a

sample	<i>M_w</i> [Da]	PDI	Z-average radius [nm]	Z-maximum PLR	PLR Z _p = 0
UFH	18,000	1.47	1110	12.8–16.0	11.2
LMWH	4300	1.37	707	2.0–3.2	3.2
PPS	5700	1.38	303	4.0–5.0	5.4
PPS Fr. 1.0	3700	1.04	284	3.2–5.0	3.2
PPS Fr. 1.1	5300	1.02	343	2.0–3.2	4.0
PPS Fr. 1.3	9100	1.01	466	3.2–4.0	8.7
PPS Fr. 1.4	11,700	1.01	506	3.2–4.0	8.3
PPS Fr. 2.0	17,000	1.02	706	5.0–6.4	11.2

^a*M_w*: molecular weight determined by HP-SEC;⁷ PDI: polydispersity index determined by HP-SEC;⁷ Z-average radius: radius value at the maximum of aggregation, Z-maximum PLR: range of PLR, determined by PCS, where the size of the complex is higher, PLR Z_p = 0: PLR at which Z_p reaches zero, as determined from the fit to the experimental data.

a colloidal interaction model. Colloidal formulations remain stable at high positive or negative charges, which prevent flocculation, whereas a neutral charge is associated with particle instability and aggregation. A positive Z_p has been linked to immunogenicity.²⁵ The surface charge of the complexes and the role of PPS and heparin ligands in complex formation could be determined by a measurement of the Z_p. The titration of PF4 with the ligands provides important information about the stoichiometry ratios and the charge. The typical titration curve is characterized by a sigmoidal curve (Figure 2). Initially, the Z_p of the protein assumes a positive value. Upon addition of the ligand, however, the Z_p shifts to negative values. At neutralization charge, where Z_p is zero, all protein molecules are assumed to be bound to ligand molecules. Comparison of the Z_p data of PPS demonstrated that it exhibits intermediate behavior between that of UFH and LMWH (Figure 2a), consistent with its intermediate *M_w*.

PPS fractions exhibited similar behavior to the unfractionated PPS sample and, as observed for heparin,²⁸ a decrease in *M_w* was strictly correlated with a decrease in the Z_p value at which charge neutralization occurred. PPS fractions 1.3 and 1.4 present similar values, hypothesized to be correlated with the slightly higher differences in the content of branched MGA residues in PPS fractions,⁸ potentially reducing the number of available sites for interaction with the protein. Higher *M_w* fractions (such as Fraction 2.0, *M_w*: 17 kDa) reached a neutral Z_p at higher log PLR than lower *M_w* fractions, indicating that lower molar ratios of the sample are required to reach protein neutralization (Figure 2b). The PLR values at which the Z_p is zero, determined from the sigmoidal fitting of Z_p versus the logarithm of the PLR, as well as the average size and corresponding PLR ranges obtained from PCS measurements, are listed in Table 1. The maximum positive Z_p values are similar between all of the PPS fractions and heparin samples (≤10 mV) but the concentrations at which these maximum values occur vary over half a log unit; PPS falling between those of LMWH and UFH (Figure 2a). Similar variation was observed in the PPS fractions (Figure 2b), indicating that again, like heparin, PPS is a heterogeneous product comprising a variety of substituents. The overall similarities between these heparin and PPS samples, particularly their positive Z_p characteristics,²⁵ suggest likely similarities in immunogenicity.

2.3. Circular Dichroism Spectroscopy

Far-UV CD (200–260 nm) spectroscopy was used to investigate changes in the PF4 secondary structure (α -helices, β -sheet, and unstructured regions) upon interaction with PPS and its fractions. For comparison, the same experiments were carried out on PF4 complexed with UFH and LMWH. Figure S2 reports the conformational changes of PF4 upon PPS binding, along with the related variations in the antiparallel β -sheet, which have been demonstrated to be a good marker for investigating structural changes of PF4/polyanion complexes.²⁹ In the presence of PPS (Figures S2, S3), the secondary structure of PF4 significantly changed with increasing ligand concentrations, as detected by the decrease of ellipticity values up to a PLR of 2. By an increase in the PPS concentration further, the ellipticity shifted toward more positive values, similar to that of native PF4. Deconvolution of the CD spectra (Figure 3a) showed the typical reversible changes in the antiparallel β -sheet content of PF4 at different PPS concentrations, and this mimics the behavior of LMWH more closely than UFH. The maximum increase in antiparallel

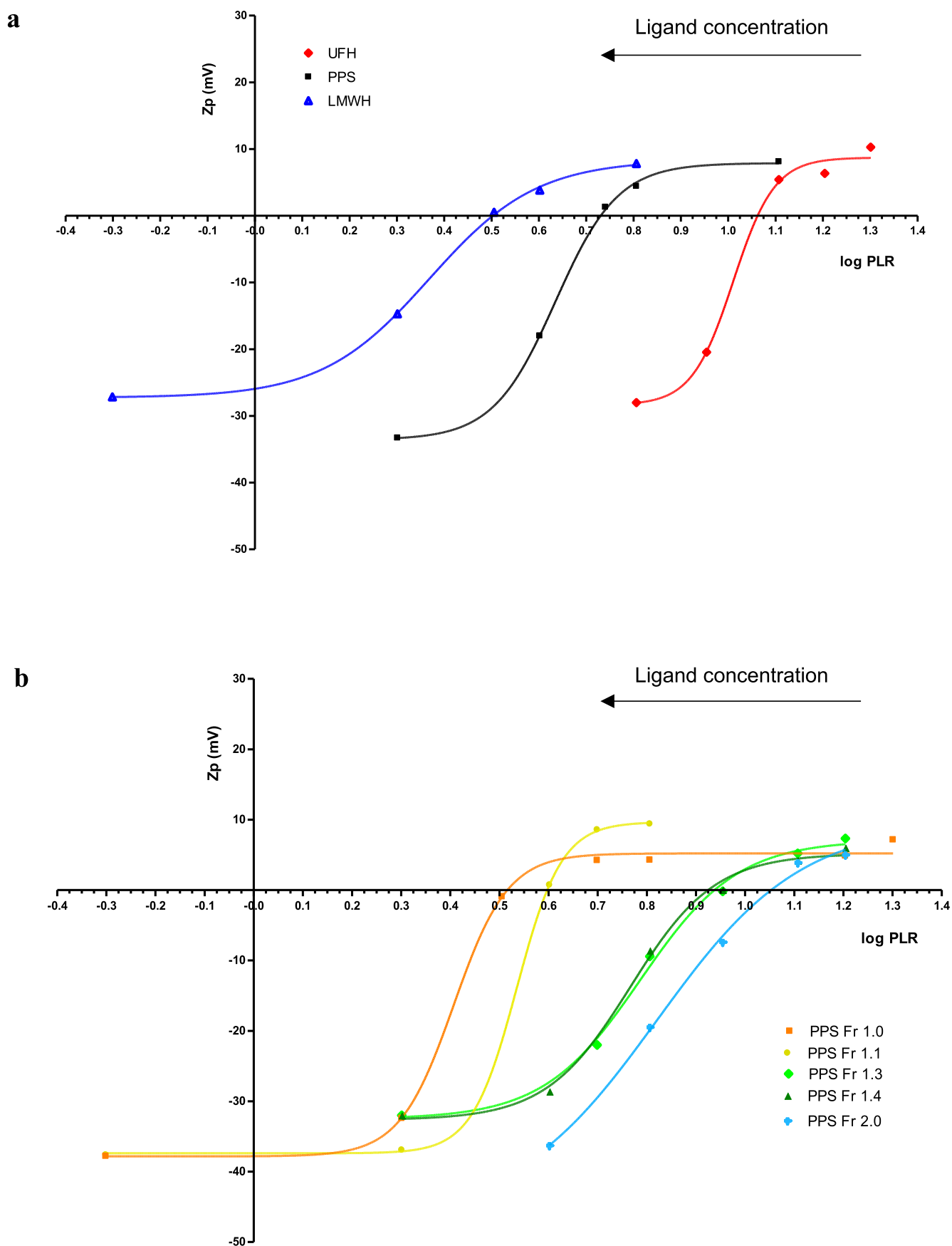


Figure 2. Surface charge of PF4/heparins or PPS complexes. Z_p average [mV] vs log PLR (PLR: protein ligand ratio) of (a) UFH (red circle solid), PPS (black circle solid), LMWH (blue circle solid); (b) PPS fractions: in detail are reported PPS Fr. 1.0 (orange circle solid), PPS Fr. 1.1

Figure 2. continued

(yellow circle solid), PPS Fr. 1.3 (green circle solid), PPS Fr. 1.4 (teal green circle solid), and PPS Fr. 2.0 (cyan circle solid). Error bars, not reported on the graphs, of Zp measurements range between 0.07 and 2.5 mV for each point based on three repetitions.

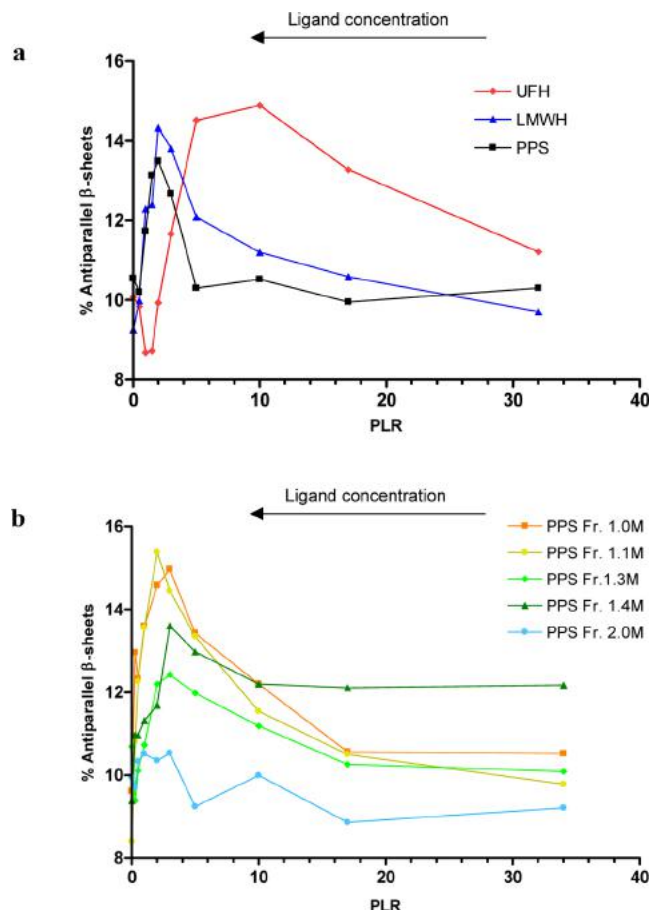


Figure 3. Changes in the PF4 secondary structure upon interaction with PPS and its fractions by CD spectroscopy. (a) Conformational changes of antiparallel β -sheets of PF4 upon addition of PPS (black circle solid), UFH (red circle solid), and LMWH (blue circle solid) or (b) in the presence of different PPS fractions, in detail are reported PPS Fr. 1.0 (orange circle solid), PPS Fr. 1.1 (yellow circle solid), PPS Fr. 1.3 (green circle solid), PPS Fr. 1.4 (teal green circle solid), and PPS Fr. 2.0 (cyan circle solid).

β -sheets occurred at similar PLR values to that of LMWH—around 2–3, while the reversible increase in antiparallel β -

sheets in the presence of UFH was measured at a PLR of about 10. The reversible changes in the PF4 β -sheet upon interaction with the ligand were observed for all PPS fractions, closely resembling those of LMWH rather than UFH; the maximal conformational changes occurring at PLR ratios between 2 and 3 (Figure 3b).

2.4. Isothermal Titration Microcalorimetry

To further characterize the binding, we applied ITC to determine the binding constants and thermodynamic parameters of interactions between PF4 and PPS and related oligosaccharides (Table 2). ITC results showed that the stoichiometry (N) of PF4 binding to each ligand was in the range of 0.9–0.13, which correlated with the different M_w and chain lengths of the samples. Indeed, the interaction of PF4 with Fraction 1.0, which contains the smallest M_w fractionated PPS, resulted in a stoichiometry of about 1, suggesting that only 1 oligosaccharide chain was bound per protein. The binding stoichiometry was, however, found to change with increasing ligand M_w ; maximum binding of about 7–8 PF4 molecules per chain occurring with PPS Fraction 2.0 ($N = 0.13$, M_w : 17 kDa). The same trend was observed for the equilibrium dissociation constant (K_D), with stronger binding for higher M_w fractions compared with the K_D of the complexes formed with a short chain length. Additionally, both PPS and its fractions had favorable enthalpic (ΔH , −13.0 to −68.2 kcal/mol) and unfavorable entropic (ΔS , 3.2–56.9 kcal/mol) terms. The negative enthalpic contribution to binding was mainly attributed to the formation of strong ionic interactions, hydrogen bonds, and van der Waals interactions, all of which increase with the chain length and consequently with the number of sulfate groups on the ligand. In contrast, the unfavorable entropic contribution was due to conformational changes restricting the conformational freedom of the complex. Similar to PPS Fraction 2.0 (M_w : 17 kDa) with comparable M_w , thermodynamic parameters were estimated for PF4/UFH binding, showing that 4 protein molecules bind per heparin chain, whereas LMWH (M_w : 4.3 kDa) showed the same stoichiometry, but weaker binding compared to the complexes formed with PPS fractions 1.0 and 1.1, which contain PPS chains with similar M_w , respectively, of 3.7 kDa and 5.3 kDa.

Table 2. Binding Constants and Thermodynamic Parameters of PF4 Interactions with PPS and Its Fractions Determined by Isothermal Titration Calorimetry (ITC)^{a,b}

sample	N (sites)	PLR (1/ N)	K_D (M)	ΔH (kcal/mol)	ΔG (kcal/mol)	$-T\Delta S$ (kcal/mol)
PPS	0.369 ± 0.003	3	$27.8 \times 10^{-9} \pm 4.86 \times 10^{-9}$	-22.9 ± 0.35	−10.3	12.6
PPS Fr. 1.0	0.899 ± 0.01	1	$74.1 \times 10^{-9} \pm 11.2 \times 10^{-9}$	-13.0 ± 0.20	−9.7	3.2
PPS Fr. 1.1	0.504 ± 0.03	2	$23.2 \times 10^{-9} \pm 3.40 \times 10^{-9}$	-20.3 ± 0.21	−10.4	9.8
PPS Fr. 1.3	0.265 ± 0.002	4	$20.2 \times 10^{-9} \pm 3.84 \times 10^{-9}$	-37.3 ± 0.56	−10.5	26.8
PPS Fr. 1.4	0.146 ± 0.001	7	$10.1 \times 10^{-9} \pm 1.77 \times 10^{-9}$	-61.3 ± 0.83	−10.9	50.4
PPS Fr. 2.0	0.132 ± 0.001	8	$5.39 \times 10^{-9} \pm 0.78 \times 10^{-9}$	-68.2 ± 0.63	−11.3	56.9
UFH	0.254 ± 0.016	4	$6.17 \times 10^{-9} \pm 0.78 \times 10^{-9}$	-47.0 ± 3.68	−11.3	35.8
LMWH	1.170 ± 0.11	1	$340 \times 10^{-9} \pm 17.7 \times 10^{-9}$	-9.20 ± 0.04	−8.83	0.37

^aResults were compared to the interactions between PF4 and UFH or LMWH. ^b N : stoichiometry of the interaction; K_D : dissociation constant; ΔH : variation of enthalpy; ΔS : variation of entropy; ΔG : variation of Gibbs free energy.

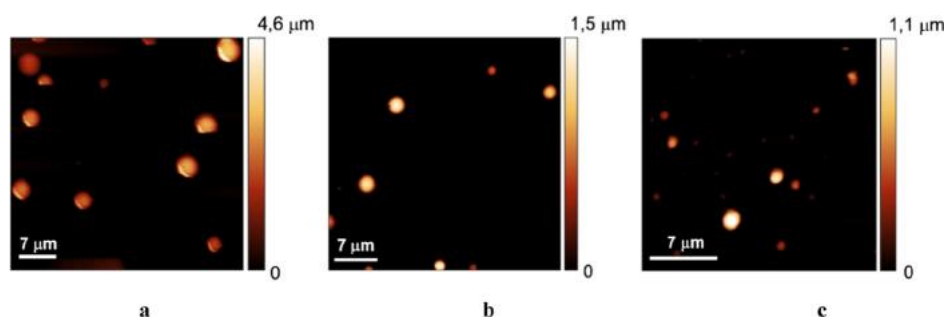


Figure 4. AFM images of the PF4/PPS aggregates, achieved at PLR close to zero Z_p of (a) PPS sample at PLR 5.4, (b) PPS fr. 1.0 at PLR of 3.2, and (c) PPS fr. 1.4 at PLR of 9.

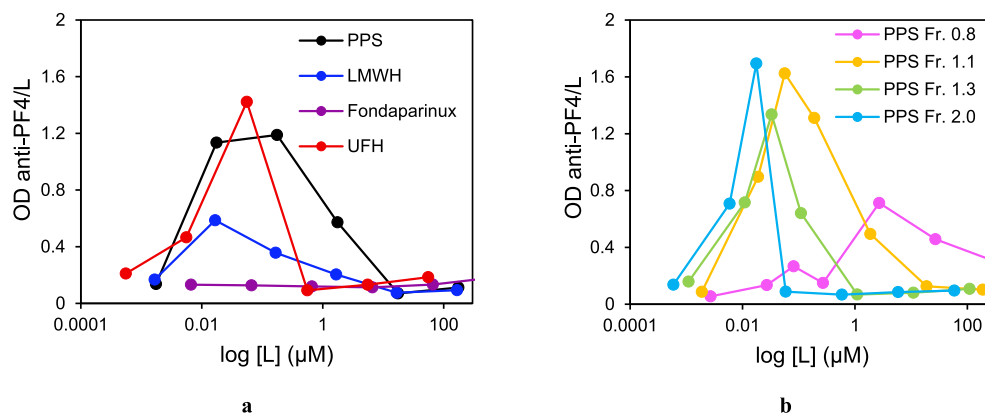


Figure 5. Reactivity of anti-PF4/ligands (L) antibody KKO. The optical density (OD) of anti PF4/L versus the logarithm of the concentration of ligands ($\log[L]$) of (a) PPS (black circle solid), UFH (red circle solid), LMWH (blue circle solid), and Fondaparinux ($M_w \sim 1.7$, purple circle solid); (b) PPS fractions; PPS Fr. 0.8 (pink circle solid), PPS Fr. 1.1 (yellow circle solid), PPS Fr. 1.3 (green circle solid), and PPS Fr. 2.0 (cyan circle solid).

2.5. Atomic Force Microscopy

Atomic force microscopy (AFM) in solution was used to characterize the PF4/PPS complexes at the nanoscale level, providing information about the shape, size, and abundance of the aggregates. Blank analysis, performed on PF4 protein deposits on mica surfaces, without the addition of the ligand, revealed an absence of aggregate formation (Figure S4). The concentration of the reagent deposited on the mica and the deposition time were both chosen in order to ensure an electrostatic interaction between the mica and the complexes. To confirm the trend obtained with the DLS data, all the samples were analyzed under conditions close to, below, and above the Z_p zero point. Table S5 shows the characteristic values measured for the PPS sample, reporting the mean aggregate volume, the percentage of aggregated material, and the average aggregate size (expressed by the equivalent sphere radius, R_{eq}), measured at three different PLR values. Similar results were observed for all of the samples investigated. Specifically, no, or few aggregates were detected at lower PLR values; the aggregate size reached a maximum at intermediate PLR values; and the degree of aggregation dropped significantly at higher PLR values.

Figure 4 shows a comparison of the AFM results for PPS, PPS fraction 1.0, and PPS fraction 1.4, obtained at PLR values close to zero Z_p , a condition previously demonstrated to represent the higher number of population aggregates. All aggregates showed approximately round structures of different sizes and abundance. The features observed in the PPS fraction 1.0 are, on average, slightly larger than those in the PPS

sample. In addition, PPS fraction 1.0 (M_w : 3.7 kDa) (Figure 4b) was characterized by higher aggregation compared to PPS fraction 1.4 (M_w : 11.7 kDa) (Figure 4c).

2.6. Enzyme Immunoassay

Enzyme immunoassay was used to quantify the formation of PF4/heparin complexes and their recognition by the monoclonal antibody KKO. The antigenicity obtained with the PF4/ligand EIA is highly concentration-dependent on the PF4/Ligand ratios. For UFH, PPS, and PPS fractions, the antigenicity rose with increasing ligand; reaching a maximum at an optimal value, and a further increase in ligand concentration decreases the binding of the specific antibody to the PF4/ligand (PF4/L) complexes. UFH, LMWH, Fondaparinux (the anticoagulant pentasaccharide, which is known not to cause HIT), and PPS comparison are reported in Figure 5a. Fondaparinux as expected does not show the formation of PF4/L antigenic complexes, while LMWH showed a strongly reduced binding in comparison to UFH and PPS samples. PF4/UFH and PF4/PPS aggregates were similar in intensity and maximum point values. Immunoassay analysis on the PPS fractions (Figure 5b) showed that PPS Fr. 2.0, the higher M_w (17 kDa), i.e., longer xylose-chains, presents a high peak at the highest molar concentrations. Otherwise, the PPS fraction with the lowest M_w (PPS fraction 0.8, M_w : 2900 Da, PDI: 1.10) showed the lowest peak of antibody binding at the lowest molar concentration (Figure 5a). This fraction shows atypical reactivity with two peaks. It also exhibits atypical behavior in physicochemical characterization and significant differences in its composition (data not shown).

3. CONCLUSIONS

Currently, pentosan polysulfate is used widely to treat bladder pain syndrome/interstitial cystitis in humans with an oral formulation. As PPS shares some structural similarities with heparin, it has also been investigated as an anticoagulant. PPS exhibits only around 1–10th the activity of heparin; however, and furthermore, it inhibits heparin action.³⁰ More recently, the potential of PPS to bind viruses, similar to the action of heparin and other GAGs, has also been explored.⁸ Several other potential applications have also been investigated, including cancer³¹ and prion diseases,³² prompting batch-to-batch analysis and comparative studies revealing that protamine sulfate neutralizes PPS more weakly than it does heparin.³³ With the interest of using PPS in a variety of new applications, some of which could include its intravenous or subcutaneous administration as well as the manufacture of materials incorporating PPS, it is also important to understand any potential risks that it may pose, and it was useful to compare these with UFH and LMWH, particularly regarding the response in relation to concentration. The major risk upon extended heparin administration is posed by the immunogenic response to complexes formed from PF4 and heparin. A fully heparinized patient may have serum concentrations of about 1.5–3.5 $\mu\text{g/mL}$ (considering a level on the serum of 0.3–0.7 IU/mL for a dose of 196 IU/mg)³⁴ and the half-life of heparin varies between 1.5 h (for UFH) to 2–5 h (for various LMWH preparations), driven in part by its enzymatic degradation by endogenous heparanase, and up to 21 h for Fondaparinux, the synthetic pentasaccharide heparin analogue. If administered intravenously, PPS would not be subject to heparanase degradation but, it is known to bind to a number of growth factors and receptors;³⁵ a half-life of 4.8 h has been reported.³⁶ The *in vivo* risk of HIT is related subtly to several structural properties that include the size of the aggregates formed, their overall charge, as well as their ability to provoke an antibody response; this latter property being further complicated as a means of predicting *in vivo* side effects by the polyclonal and polyspecific nature of the antibody response.³⁷ The size of PPS/PF4 complexes was found to depend on their molar ratios and was intermediate between those formed by PF4 with UFH and LMWH but its maximum size was smaller than that formed with heparin. More complex behavior was observed with the PPS fractions, although the complexes were smaller with lower-molecular-weight PPS fractions. Zeta potential analysis provided deeper insights into the behavior of PF4/PPS aggregates by enabling the determination of the zero point, which corresponds to maximum complex formation.²⁸ PPS exhibited a comparable profile, and as for PCS measurements, its extrapolated Zp zero value lay between those of LMWH and UFH (Table 1), consistent with the M_w of the PPS samples. This trend was also observed in the PPS fractions, where the lowest zero Zp value was found in the fractions with the lowest M_w (PPS Fr. 1.0, M_w 3.7 kDa). This indicates that more ligand is required to reach the neutralization point. The CD spectra revealed reversible secondary structural changes in PF4 upon the addition of UFH, LMWH, PPS, and its fractions at high concentrations.³⁸ In addition, PF4/PPS exhibited intermediate behavior between that of UFH and LMWH. The maximum increase in antiparallel β -sheets was observed at a PLR value of 2, suggesting that two PF4 molecules bind to each PPS chain. Studies using ITC also showed that PPS exhibits PF4 affinity values between those of UFH and

LMWH. Its stoichiometry (0.37) indicates that two to three PF4 proteins are bound to each PPS chain. Binding interactions were exothermically and enthalpically driven in all samples, suggesting the formation of strong ionic interactions coupled with hydrogen bonds and van der Waals interactions between the sulfate groups of the ligand and PF4. For both PPS and UFH, complex formation was accompanied by negative entropy, indicating the restriction of the conformational freedom of the ligands and conformational rearrangements of the protein. ITC studies of PPS fractions showed that the binding affinity increases with the molecular weight of the PPS fractions.

The relatively low K_D values achieved for PF4 with PPS and its fractions were indicative of a high affinity. The higher negative enthalpy seen in PF4/PPS interactions can be attributed mainly to the formation of strong ionic, hydrogen bond, and van der Waals interactions with the positively charged PF4 protein.

AFM confirmed that aggregation depends on the ligand concentration. Smaller particles adhered more easily to the mica surface, and the limited scanning range favored the detection of smaller- and medium-sized subpopulations while excluding the larger components (size greater than, approximately, 5–6 μm), which were observed by optical microscopy (data not shown). These limitations of the AFM technique explain why PPS Fraction 1.0 aggregates were larger than those of PPS Fraction 1.4 (M_w = 11.7 kDa). PPS samples exhibit different sizes, confirming these observations.

Finally, immunoassay analysis of KKO antibody binding to both PF4–heparin and PF4/PPS complexes was consistent with other analytical data and binding was strongly dependent on PLR.²⁷ The size, approximately 12 sugar units, was identified as a key parameter of interaction in this study. While PPS showed antigenicity closer to UFH than to LMWH, despite its M_w resembling that of LMWH, the antigenicity of the PPS fractions was dependent on the chain length of the sugar backbone. The *in vitro* detection of HIT-antibody binding does not, however, strictly correlate with *in vivo* immunogenicity or clinical outcome, as antibodies against PF4–ligand complexes, especially with UFH, are more common than active antibodies in functional assays and, these are not always associated with clinical signs of HIT. Furthermore, Fondaparinux, based on a single pentasaccharide from heparin, cannot induce the neoantigen on PF4 and therefore exhibits no *in vitro* binding of HIT-2 antibodies in diverse assays but has been shown to induce anti-PF4/ligand antibodies in patients treated solely with Fondaparinux.³⁹ Overall, the findings suggest that regarding the possible induction of HIT through interactions with PF4, PPS exhibits properties comparable to those of heparin (UFH) and its derivatives (LMWH). To ensure that any future applications of PPS, especially involving intravenous application, are safe, further studies, particularly *in vivo*, are warranted.

Interestingly, despite PF4 complexes with PPS, and PF4 complexes with its fractions being smaller than those formed between PF4 with UFH or LMWH, enzyme immunoassay studies demonstrated the formation of antigenic (KKO antibody) complexes. It is recognized that the KKO antibody mimics the behavior of some HIT antibodies; however, not all antibodies and further detailed investigation of the immunogenicity of PPS and its fractions will be necessary. Since PPS appears to provoke comparable interactions with PF4 and has a plasma half-life longer than heparin, but much shorter than

Fondaparinux, the results suggest that monitoring of potential thrombocytopenia effects will be necessary (as for treatment with heparin) when considering PPS dosing, especially for intravenous applications.

The current state of knowledge concerning the structural causes underlying the propensity of PPS to induce HIT does not provide an explanation of the precise mechanism nor suggest particular structural or biochemical characteristics that can provide proxy measures, or serve as predictors, of its extent. With this limitation in mind, but wary of the fact that PPS is employed as an anticoagulant, we emphasize that similar structural and immunological consequences arise through PPS interactions with PF4 as with heparin, whose tendency to induce HIT in patients is well known. The use or any future development of PPS for indications in which its intravenous administration is proposed must, therefore, be tempered by an appreciation of the possibility of a comparable risk of inducing HIT.

4. MATERIALS

The pentosan polysulfate and the six fractions (named PPS Fraction 1.0, Fraction 1.1, Fraction 1.3, Fraction 1.4, and Fraction 2.0 at different molecular weights (M_w),⁸) used in this study were supplied by bene pharmaChem GmbH & Co.KG (Geretsried, Germany). Low-molecular-weight heparin (LMWH, Enoxaparin) was purchased from Sanofi Aventis (Bridgewater, New Jersey, United States), while for enzyme immunoassay, the LMWH Deltaparin, purchased from Pfizer Pharma (Berlin, Germany), was used. Fondaparinux was purchased from Mylan IRE Healthcare (Dublin, Ireland). Unfractionated porcine heparin (UFH) was purchased from Hepalink Pharmaceutical Co. (Shenzhen, China); UFH used for enzyme immunoassay was purchased from Ratiopharm (Ulm, Germany). Hank's balanced salt solution was purchased from Sigma-Aldrich (Milan, Italy), while phosphate-buffered saline was purchased from Biowest (France). 96-well Maxisorp F8 plates were purchased from Thermo Scientific (Dreieich, Germany). PBS-Tween was purchased from Jackson ImmunoResearch (Baltimore Pike, United States). Human-platelet factor 4 (PF4) freeze-dried in HBSS was purchased from ChromaTech (Greifswald, Germany). Lyophilized PF4 was dissolved in prefiltered water to achieve a concentration of 1 mg/mL (32 μ M), while for enzyme immunoassay it was solubilized at a concentration of 3 μ g/mL. PF4Monoclonal Antibody (KKO, MA5-17,641) were purchased from ThermoFisher Life Technologies GmbH (Darmstadt, Germany).

5. METHODS

5.1. Photon Correlation Spectroscopy

Photocorrelation spectroscopy (PCS) was used to determine the size of the particles in solution. PCS measurements were performed with a DLS Zetasizer Nano ZS (Malvern Instruments Ltd., UK) with a fixed angle at 173° and a 633 nm helium–neon laser, and the data were analyzed with Zetasizer software version 7.12. To evaluate aggregate formation, the protein was titrated at different protein/ligand ratios (PLRs). For each sample, five different points were evaluated. Size solutions were measured after the transfer in a disposable size cell (ZEN0040, Malvern Analytical, UK) of 80 μ L of a solution, prepared as reported by Bertini et al. 2017,²⁸ obtained by the addition of a suitable volume of HBSS, ligand sample solution at different concentrations, and 4 μ L of PF4 32 μ M. The analyses were recorded after 60 min at room temperature (RT).

5.2. Zeta Potential

The Z_p was measured with a Zetasizer Nano ZS (Malvern Instruments Ltd., UK), and the data were analyzed with Zetasizer software version 7.12. Origin Software was used to fit the Z_p data versus the logPLR, allowing determination of the neutral state of the complex ($Z_p = 0$). As for PCS, for each sample, at least 5 different

points were evaluated. In detail, to analyze the Z_p , 40 μ L of PF4 32 μ M was added to the ligand sample solution and diluted with the appropriate volume of water. Measurements were performed after 10 min of incubation at RT in a disposable folded capillary cell (DTS1070, Malvern Analytical, UK).²⁸

5.3. Circular Dichroism Spectroscopy

Circular dichroism spectroscopy was used to determine the changes in the protein structure that occurred after the interaction with ligands. Spectra (200–260 nm) were recorded using a J-1500 CD Spectrometer (Jasco, Japan), and data were analyzed by Jasco Spectra Manager Software v2.14.02. The solution of PF4 (32 μ M) was diluted in PBS (0.138 M NaCl, 0.0027 M KCl, pH 7.4) to a concentration of 1.28 μ M. Spectra were recorded at 25 °C in a 1 cm optical path length quartz cell (Helma, MI, Italy). Ten different PLRs were evaluated, starting with the measurement of 200 μ L of PF4 alone, followed by increasing additions of the PPS sample in the cuvette. For each sample, a baseline was performed, acquiring and subtracting a CD spectrum for each sample concentration point without the addition of the PF4 protein.

5.4. Isothermal Titration Calorimetry

Thermodynamic properties, such as variation of enthalpy, entropy, and Gibbs free energy (ΔH , ΔS , and ΔG) and the binding affinities between samples and PF4, were obtained by ITC and were carried out using a MicroCal Peaq-ITC (Malvern Analytical, UK). To perform ITC measurements, 200 μ L of 10 μ M PF4 solution was loaded in the sample cell, and heparin or PPS solution was added into the injection syringe. Each ITC experiment consists of 20 injections of 2 μ L each with a delay of 180 s between injections; the stirring rate was set to 500 rpm, and the temperature was set to 25 °C. To estimate the thermodynamic parameters (KD, ΔH , and ΔS), the data were fitted using the MicroCal analysis software.

5.5. Atomic Force Microscopy

Atomic force microscopy analyses were performed using an NX-12 microscope (Park Systems, Sud Korea) equipped with a Nikon IX optical microscope. Characterization was carried out in noncontact mode using qp-BioAC probes (Uniqprobe, CH) with a nominal elastic constant of 0.3 N/m and a resonance frequency of 90 kHz. The real elastic constant of the cantilever was regularly checked through the thermal noise method. For each sample, at least three different PLRs were evaluated. AFM samples were prepared on cleaved mica (1 cm²) by depositing 80 μ L of solution (distilled water and PPS solution), followed by 40 μ L of PF4 (32 μ M). After 10 min of incubation at 20 °C, 3 mL of distilled water was used to remove unbound complexes, and the samples were analyzed immediately. During acquisition, the driving frequency was set between 50 and 100 kHz and with a cantilever oscillation amplitude kept below 20 nm. For each PLR, at least 10 images were collected from different random areas (including 5 μ m-wide images collected with a 2–4 s/line scan speed). The data were collected using the SmartScan software (Park Systems, Sud Korea), while the analysis was conducted with the open software Gwyddion (v. 2.59), considering complexes larger than 20 nm. The observed nanostructures were divided in small-, medium-, and large-size populations, and the equivalent sphere radius (R_{eq}) was calculated from the measured average volume of each population.

5.6. Enzyme Immunoassay

Platelet factor 4/ligand enzyme immunoassay (EIA) was performed with monoclonal antibodies (mab, a mouse IgG antibody) known to react specifically with PF4/heparin complexes and to induce HIT in platelet activation tests as described in the literature, called KKO.²⁶ PF4 (3 μ g/mL) was preincubated with rising concentrations of ligand in PBS buffer for 60 min at RT to enable complex formation, before coating wells of a microtiter plate with 100 μ L at RT for further 60 min. Then, plates were washed three times with PBS-Tween (0.15 M NaCl, 0.1% Tween 20, pH 7.5) and incubated with 200 μ L of blocking solution for 60 min at RT (Roti-block, Roth, Karlsruhe, Germany). Then, the plates were washed three times with PBS-Tween and incubated with 100 μ L of diluted mAb for 60 min (KKO,

diluted to 0.1 $\mu\text{g/mL}$ in PBS-Tween). Plates were washed three times and incubated with 100 μL of peroxidase-conjugated antimouse IgG diluted 1:20,000 in PBS-Tween. Afterward, plates were washed four times and incubated (10 min, RT) with 100 μL of TMB (tetramethylbenzidine). The reaction was stopped by adding 100 μL of 1 M H_2SO_4 and absorbance was measured at 450 nm.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.5c11319>.

Structure of PPS (Figure S1); deconvolution of PF4/ligand circular dichroism spectra for PPS, LMWH, UFH, and PPS fractions (PPS Fr. 1.0, PPS Fr. 1.1, PPS Fr. 1.3 PPS Fr. 1.4, and PPS Fr. 2.0) (Figure S2); CD spectra showing the changes in ellipticity upon addition of PPS at different PLR (protein ligand ratio) (Figure S3); AFM analysis of PF4, without the addition of ligand (Figure S4); and AFM subpopulation of PPS/PF4 complexes observed at different PLR (Table S5) (PDF)

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Sofia Nizzolo: investigation; methodology; conceptualization; writing—original draft; writing—review and editing. Serena Zanzoni: investigation; writing—original draft; writing—review and editing. Hans-Peter Holthoff: investigation; methodology. Marco Girasole: writing—review and editing. Rudolf Gruber: investigation; writing—original draft; writing—review and editing. Dominik Lenhart: conceptualization; investigation; writing—review and editing. Ed Yates: conceptualization; writing—review and editing. Marco Guerrini: conceptualiza-

tion; writing—review and editing. Sabrina Bertini: conceptualization; project administration; supervision; writing—review and editing.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS LIST:

AFM	atomic force microscopy
CD	circular dichroism spectroscopy
DLS	dynamic light scattering
EIA	enzyme immunoassay
EMA	European Medicines Agency
FDA	Food and Drug Administration
HIT	heparin-induced thrombocytopenia
ITC	isothermal titration calorimetry
LMWH	low-molecular-weight heparin
M_w	molecular weight
PCS	photon correlation spectroscopy
PF4	platelet factor 4
PLR	protein (PF4)/ligand ratio
PPS	pentosan polysulfate
UFH	unfractionated heparin
Zp	zeta potential

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