Complex formation with nucleic acids and aptamers alters antigenic properties of platelet factor 4

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Key Points

- PF4 binds to nucleic acids and thereby exposes the epitope to which anti-PF4/heparin antibodies bind
- PF4/aptamer complexes can induce an immune response resembling heparin-induced thrombocytopenia

Abstract

The tight electrostatic binding of the chemokine platelet factor 4 (PF4) to polyanions induces heparin-induced thrombocytopenia (HIT), a prothrombotic adverse drug reaction caused by immunoglobulin G directed against PF4/polyanion complexes. This study demonstrates that nucleic acids, including aptamers, also bind to PF4 and enhance PF4 binding to platelets. Systematic assessment of RNA and DNA constructs, as well as four aptamers of different lengths and secondary structures, revealed that increasing length and double-stranded segments of nucleic acids augment complex formation with PF4, while single nucleotides or single-stranded poly A or poly C constructs do not. Aptamers were shown by circular dichroism spectroscopy to induce structural changes in PF4 that resemble those induced by heparin. Moreover, heparin-induced anti-human-PF4/heparin antibodies cross-reacted with human PF4/nucleic acid- and PF4/aptamer complexes, as shown by an enzyme-immunoassay and a functional platelet activation assay. Finally, administration of PF4/44mer-DNA Protein C-aptamer complexes in mice induced anti-PF4/aptamer antibodies, which cross-reacted with murine PF4/heparin complexes. These data indicate that the formation of anti-PF4/heparin antibodies in postoperative patients may be augmented by PF4/nucleic acid complexes. Moreover, administration of therapeutic aptamers has the potential to induce anti-PF4/polyanion antibodies and a prothrombotic diathesis.
Introduction

The chemokine platelet factor 4 (PF4) is released from platelet α-granules during platelet activation, and binds, due to its high positive charge, to many negatively-charged polyanions, including heparin. PF4 forms large multimolecular complexes with heparin that are highly immunogenic. The resulting immunoglobulin G (IgG) antibodies are the cause of heparin-induced thrombocytopenia (HIT), a prothrombotic adverse drug effect. In HIT, multimolecular complexes comprised of PF4, heparin, and anti-PF4/heparin IgG cross-link platelet FcγIIa receptors, triggering platelet activation, microparticle formation, and thrombin generation, with ~50% of affected patients developing thrombosis.

Recently, we showed that PF4 binds to polyanions on the surface of bacteria, thereby forming multimolecular complexes that are recognized by human anti-PF4/heparin antibodies. More specifically, we identified the PF4 binding site on Gram-negative bacteria as the phosphate groups of lipid A. Based on this observation, we hypothesized that nucleic acids might also form multimolecular complexes with PF4 because they also expose multiple negatively-charged phosphate groups. This idea was fostered by previous findings that salmon sperm DNA could substitute heparin in HIT-IgG induced platelet activation.

Plasma levels of extracellular nucleic acids are regulated by prompt nuclease degradation and renal clearance, resulting in their short half-life (4-30min), with a slightly longer half-life for DNA than for RNA. In healthy individuals, extracellular nucleic acid concentrations in plasma range from 0 to >1000ng/mL. However, under pathological conditions, cell-free nucleic acids can be generated from breakdown of bacteria and viruses, tissue damage, cell apoptosis, and release from blood cells such as formation of neutrophil extracellular traps (NETs). Plasma levels of extracellular nucleic acids can rise up to 2,000µg/mL.
With the recent introduction of aptamers, small DNA/RNA constructs designed as therapeutic oligonucleotides, the interaction of nucleic acids and PF4 becomes more relevant from a drug safety perspective. In this study, we characterize the structural features of RNA and DNA molecules, as well as aptamers, that are relevant for complex formation with PF4. We also demonstrate by circular dichroism (CD) spectroscopy that nucleic acids cause conformational changes in PF4 similar to those induced by heparin, and show that PF4/aptamer complexes can be highly immunogenic.
Methods

Platelets and sera

Platelet-rich plasma was prepared from 10mL hirudinized whole blood (10µg/mL lepirudin, Pharmion, Hamburg, Germany), obtained from healthy blood donors (120g, 20min, 30°C). Platelets were isolated by gel-filtration as described14 and adjusted to 50×10⁹/L in Tyrode buffer (137mMNaCl, 2.7mMKCl, 2mM MgCl₂x6H₂O, 2mM CaCl₂x2H₂O, 12mM NaHCO₃, 0.4mM NaH₂PO₄, 0.4% BSA, 0.1% glucose, pH 7.2). Sera containing anti-PF4/heparin antibodies were leftovers from clinical specimens used for laboratory diagnosis of HIT.

Nucleic acids and aptamers

Sequences and sources of aptamers and nucleic acid compounds are given in Figure-1. Secondary structures were calculated by the mFold RNA and mFold DNA database of the University of Albany (N.Y., USA, http://mfold.rna.albany.edu/?q=mfold). The single-stranded 15mer-DNA thrombin aptamer targets thrombin;15 the 44mer-DNA protein C aptamer inhibits activated protein C;16 the 57mer-RNA tetracycline aptamer binds tetracycline;17 and the 77mer-RNA flavin mononucleotide (FMN) aptamer is an artificial FMN-responsive catalytic RNA.18 Cellular RNA and DNA were isolated from mouse vascular smooth muscle cells (MVSMC) using an extraction kit (Sigma, Munich, Germany) or the DNazol reagent (Invitrogen, Groningen, Netherlands).
**Binding of biotinylated RNA to PF4**

Microtiter plates were coated with 50 µL solutions of PF4 or bovine serum albumin (10µg/mL each) in 100mM sodium carbonate (pH 9.5) at 4°C for 20h; washed and blocked (TBS, 3% BSA; 2h). Different concentrations of biotinylated RNA (0.78-25µg/mL), prepared from isolated cellular RNA using the Psoralen-PEO-Biotin reagent (Thermo Fisher Scientific [Pierce], Rockford, N.Y.), were allowed to bind at 22°C for 2h, followed by three washes with TBS. Bound biotinylated RNA was detected using peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark) and the immunopure 3,3’,5,5’-tetramethylbenzidine (TMB) substrate kit (Pierce). In competition experiments, increasing concentrations of biotinylated RNA were mixed with unlabeled RNA, DNA or heparin (each at 300µg/mL) before binding assays were performed. To confirm that PF4 was not removed from the plate upon heparin addition, surface-coated PF4 was quantified by dot blot analysis and densitometry.

**Influence of nucleic acids on PF4 binding to platelets**

The impact of nucleic acids on binding of human platelet-derived PF4 (25µg/mL) (ChromaTec, Greifswald, Germany) to gel-filtered platelets (50,000/µL) was assessed by flow cytometry (Cytomics FC 500, Beckman Coulter, Krefeld, Germany) using a polyclonal FITC-labeled rabbit anti-human PF4 antibody (Dianova, Marl, Germany), in the absence or presence of unfractionated heparin (UFH; activity ~150IU/mg; Braun, Melsungen, Germany) or different nucleic acid compounds (0.08-640µg/mL; Figure 1). In parallel, RNA samples were treated with RNase A (100µg/mL, 90min, 37°C) (Fermentas, St. Leon-Rot, Germany) before addition to the respective test solution to document RNA dependency. Antibody binding was quantified by geometric mean fluorescent intensity.
**PF4/nucleic acid enzyme immunoassay (EIA)**

Binding of human anti-PF4/heparin IgG to PF4/nucleic acid complexes was assessed by EIA\(^\text{19}\) using microtiter plates (Nunc, Langenselbold, Germany) coated (16h; 4°C) with preformed PF4/polyanion complexes: PF4 (20µg/mL) preincubated (1h, room temperature) with either UFH as positive control (3.33µg/mL), 15mer-DNA thrombin aptamer, 44mer-DNA protein C aptamer, 57mer-RNA tetracycline aptamer, or 77mer-RNA FMN aptamer in concentrations ranging from 1.25-80µg/mL. To find the optimal stoichiometric ratios for complex formation, we first titrated selected aptamers in the presence of a constant PF4 concentration in the EIA. As control for specific antibody binding, UFH (660µg/mL) was added to disrupt PF4/polyanion complexes and to inhibit antibody binding.

**Platelet activation assay**

Platelet activation by human anti-PF4/polyanion antibodies was assessed by the heparin-induced platelet activation (HIPA) assay, as described,\(^\text{20}\) using 0.2µg/mL heparin (positive control: reviparin; Abbott GmbH&Co. KG, Wiesbaden, Germany), deoxynucleotidetriphosphates (0.05-1,000µg/mL), 21mer-double-stranded DNA (0.05-1,000µg/mL), 21mer-hairpin DNA (0.05-1,000µg/mL), cellular RNA (0.05-5µg/mL), 15mer-DNA thrombin aptamer (0.5-40µg/mL), 44mer-DNA protein C aptamer (0.05-1,000µg/mL), 57mer-RNA tetracycline aptamer (0.5-5 µg/mL), and 77mer-RNA FMN-ribozyme (0.5-5µg/mL). To find the optimal concentrations for platelet activation, titration with the respective nucleic acid constructs in the HIPA test with one or two sera was performed, and then additional sera were assessed using this concentration. Specificity of platelet activation was confirmed by its inhibition using high concentrations of heparin (660µg/mL) that disrupts
PF4/polyanion complexes,\textsuperscript{21} as well as the monoclonal antibody IV.3 (2.5µg/mL; LGC Promochem, Wesel, Germany), which blocks FcγIIa receptor-mediated platelet activation.\textsuperscript{22}

**Circular dichroism (CD)-spectroscopy of PF4/polyanion complexes**

Conformational changes of PF4 (40µg/mL) were measured by far UV CD-spectroscopy (200-260nm; Chirascan CD spectrometer; Applied Photophysics, Leatherhead, UK) at increasing concentrations of heparin (6.66-73.33µg/mL), or 44mer-DNA protein C aptamer (0.31-40µg/mL) in a 101-QS quartz precision cell (path length: 10mm) (Hellma, Müllheim, Germany). Baseline spectra of PBS buffer, heparin, and 44mer-DNA protein C aptamer were subtracted to estimate secondary structures of the respective PF4/heparin or PF4/aptamer complexes (CDNN CD Spectra Deconvolution Software).\textsuperscript{23}

**Immunization of mice by PF4/aptamer complexes**

C57BL/6 mice (8–10 weeks of age; Charles River Laboratories Europe, Kisslegg, Germany) were anesthetized (2% xylazin/10% ketamin) and immunized as described\textsuperscript{24} with recombinant murine PF4 (mPF4) (ChromaTec, Greifswald, Germany), 44mer-DNA protein C aptamer, mPF4/44mer-DNA protein C aptamer complexes (each at 200µg/mL), or complexes of mPF4 (200µg/mL) and heparin (33µg/mL) in a final volume of 100µL via retroorbital injection daily for five days. After another 10 days, blood was withdrawn and serum was analyzed for anti-mPF4/aptamer- or anti-mPF4/heparin antibodies, respectively, by EIA using mPF4/heparin and mPF4/44mer-DNA protein C aptamer complexes as antigens.
Ethics

All blood donors gave informed consent. The use of antibodies from leftovers of clinical diagnostic material was approved by the ethical board of the Greifswald University. The animal studies were performed and approved according to the regulations of the Greifswald University.

Statistical analysis

Binding of biotinylated RNA to PF4 in the presence of competing polyanions was compared using paired samples t-test. The significance of differences in the impact on PF4 binding to platelets between nucleic acids was tested by analysis of variance (ANOVA) and paired samples t-test (RNase experiment). Reduction of antibody binding in the PF4/polyanion immunoassays after the heparin inhibition step was assessed by paired samples t-test. Murine immune responses to mPF4/44mer-DNA protein C aptamer complexes and mPF4 were compared by Wilcoxon two-sample test. The significance level was .05.
Results

RNA binds to PF4

Binding of RNA to PF4 was assessed using biotinylated RNA, which bound to PF4 in a saturable manner, reaching a maximum at 6.25µg/mL (Figure-2). High concentrations of unlabeled RNA, DNA, or heparin decreased binding of biotinylated RNA to PF4, with heparin showing the strongest inhibitory effect, followed by RNA and DNA. This indicates charge-dependent binding of RNA to PF4.

RNA and DNA enhance PF4 binding to platelets

Next we assessed the interaction of PF4 and different nucleic acid constructs with gel-filtered platelets by flow cytometry, as previously described\textsuperscript{14} for PF4-polysaccharide-platelet interactions. To analyze the structure-function relationship, RNA and DNA constructs of different length and structural complexity were tested. Figure-3 shows the influence of these constructs on PF4 binding to the platelet surface, whereby baseline binding was defined as PF4 binding to platelets in the absence of nucleic acids. Heparin, the prototype compound for PF4-polyanion interaction, increased PF4 binding to platelets in a dose-dependent manner, represented by a bell-shaped curve with a maximal 4.44-fold increase (±0.15, shown as grey curve in Figure-3A-D).

Cellular RNA exhibited a similar effect on PF4 binding to platelets as heparin, with a maximal 3.69-fold increase (±1.09, Figure-3A) at 0.63µg/mL, which was almost completely abrogated by pretreatment with RNase A (1.25-fold increase±0.29; \( P=\,0.0037, \, n=3 \); Figure-3A).
Enhancement of PF4 binding to the platelet surface by DNA or RNA was dependent on the size of the nucleic acid construct: shorter constructs (10mer-single-stranded DNA; 21mer-hairpin RNA) showed a weaker effect and required higher concentrations than longer constructs (45mer-double-stem-loop RNA II; Figure-3B). RNA-compounds appeared to have a stronger effect on PF4 binding than DNA-homologues, as exemplified by the 45mer-double-stem-loop RNA I, which induced binding of more PF4 to platelets, compared to the 45mer-double-stem-loop DNA ($P<.0001$; Figure-3C), although both 45mers had the same nucleotide sequence (RNA, however, contained uridine and ribose instead of thymidine and deoxyribose).

In contrast to the structured 45mers, the unpaired homopolymers 45mer-polyA and 45mer-polyC, or single nucleotides did not promote PF4 binding to platelets (Figure-3C). Furthermore, the influence of three DNA 21mers with distinct secondary structures (Figure-1B-D) on PF4 binding was analyzed: The 21mer-double-stranded DNA showed the strongest enhancement on PF4 binding to platelets ($P<.0001$, Figure-3D), while the two single-stranded constructs were far less effective. PF4 binding increased with the structural complexity of the nucleic acid construct (compare 21mer-hairpin vs. 21mer-single-stranded DNA, $P<.0001$, Figure-3D), indicating that interactions of nucleic acids with PF4 were not only charge-dependent but determined by their conformation.

**Aptamers enhance PF4 binding to platelets**

We then investigated the influence of four different aptamers (Figure-1A) on PF4 binding to the platelet surface. All four aptamers enhanced PF4 binding to gel-filtered platelets; however, maximal PF4 binding in each case occurred at different concentrations of the
respective aptamer: The 57mer-RNA tetracycline aptamer (2.48-fold increase±0.63; Figure-4A) and the 77mer-RNA FMN aptamer (2.07-fold increase±0.52; Figure-4B) induced maximal PF4 binding to platelets at similar concentrations (0.63µg/mL). However, maximal enhancement of PF4 binding by these aptamers was lower than that induced by heparin. The 44mer-DNA protein C aptamer induced maximal enhancement of PF4 binding (2.59-fold increase±0.35) at a greater than 10-fold higher concentration of 5µg/mL (Figure-4C), whereas the short 15mer-DNA thrombin aptamer enhanced PF4 binding only at very high concentrations (at 320µg/mL: 3.57-fold increase±1.1; Figure-4D).

Aptamer-induced changes in PF4 resemble those induced by heparin

The impact of nucleic acids on the secondary structure of PF4 was analyzed using CD-spectroscopy. The 44-mer-DNA protein C aptamer was used due to its nuclease-resistance. Although changes in the PF4 structure induced by complex formation with the 44-mer-DNA protein C aptamer were similar to those induced by heparin, the most pronounced alterations of PF4 were observed at broader ranges of 44mer-DNA protein C aptamer (5-20µg/mL) compared with that seen with heparin (6.9–7.2µg/mL; Figure-5A).

Deconvolution of the spectra of PF4 obtained after addition of 5µg/mL 44mer-DNA protein C aptamer or 6.9µg/mL heparin, respectively, showed an increase in anti-parallel β-sheet content from 24.2% to 34.3% for heparin and to 35.8% for the 44mer-DNA protein C aptamer. This refolding was balanced by a decrease of the α-helix fraction from 12.2% to 8.9% (heparin) and to 8.1% (44mer-DNA protein C aptamer) and a decrease in the β-turn fraction from 22.8% to 19.8% (heparin) and 20.5% (44mer-DNA protein C aptamer). Parallel β-sheet and random coil content did not change significantly.
Human anti-PF4/heparin antibodies bind to PF4/nucleic acid complexes

To assess whether complex formation between aptamers and PF4 also results in exposure of the same epitopes as on PF4/heparin complexes, we tested human sera known to contain anti-PF4/heparin IgG by EIA. Antibody binding to PF4/heparin (positive control) resulted in a mean OD 1.130±0.143. Anti-PF4/heparin antibodies bound differently to complexes formed by PF4 and distinct aptamers. They reacted only very weakly with PF4/15mer-DNA thrombin aptamer complexes (at 40µg/mL: mean OD 0.431±0.064, Figure-5D), but strongly with PF4/44mer-DNA protein C aptamer complexes over a broad concentration range (5–30µg/mL aptamer/20µg/mL PF4; mean OD 1.098±0.140, Figure-5D). Binding was significantly reduced by addition of high concentrations of heparin (mean OD 0.593±0.203, P<.0001, Figure-5D). (Marked inhibition of antibody binding against PF4/polyanion complexes at high concentrations of heparin is a characteristic feature of anti-PF4/polyanion antibody binding.)

Less pronounced binding was observed to PF4/57mer-RNA tetracycline aptamer complexes (at 20µg/mL: mean OD 0.819±0.115, Figure-5D), and PF4/77mer-RNA-FMN aptamer complexes (at 10µg/mL: mean OD 0.910±0.190, Figure-5D). Control sera did not react with any PF4/nucleic acid complexes (OD range 0.02–0.28, n=11, data not shown in the Figure).

Human anti-PF4/heparin antibodies induce platelet activation in the presence of nucleic acids and aptamers

Among 29 human sera that caused platelet activation in the presence of heparin, 18 (62.1%) also caused platelet activation in the presence of nucleic acids. These 18 sera induced platelet aggregation in the presence of all nucleic acid constructs, including aptamers, but not in the presence of buffer. Mean lag-time to platelet activation was slightly longer for the nucleic
acid constructs (12.69±2.84 minutes) than for heparin (10.73±6.27 minutes), with only minor
differences between the single constructs (Figure-5B). Mean reactivity with donor platelets
(i.e. the platelets of how many donors reacted) was also lower for the nucleic acids (85.31%)
than for heparin (93.22%), with the 15mer-DNA thrombin aptamer showing lowest reactivity
(Figure-5C). Platelet aggregation was consistently inhibited by high concentrations of heparin
as well as by the moAb IV.3.22 In contrast to heparin, the nucleic acid constructs induced
platelet aggregation over a wide concentration range (0.005–100µg/mL). Consistent with
what is known from heparin, very high concentrations of nucleic acids exceeding 1,000µg/mL
inhibited platelet aggregation. None of the control sera (n=9) induced platelet activation in the
presence of heparin or any nucleic acid construct.

**PF4/aptamer complexes induce anti-PF4/polyanion antibodies in vivo**

In mice mPF4/44mer-DNA protein C aptamer complexes induced a strong and robust
immune response within 15 days in all animals (median OD 2.39, range 1.96-2.61, n=5,
Figure-5E). These antibodies cross-reacted against mPF4/heparin complexes (median OD
1.73, range 1.10-2.13, n=5, Figure-5E). Mice immunized with mPF4/heparin complexes
(positive control) showed an immune response against mPF4/heparin complexes (median OD
1.58, range 0.61-2.76, n=7). Injection of the aptamer alone did not induce antibody formation
with the exception of one animal (median OD 0.20, range 0.10-1.39, Figure-5E).
Immunization with mPF4 alone also caused an immune response (median OD 0.84, range
0.32-2.52; Figure-5E), but this was significantly weaker than the immunization induced by
mPF4/44mer-DNA protein C aptamer complexes (P=.015). The reason for an immune
response to mPF4 alone is most likely formation of some mPF4-autoaggregates resembling
mPF4/aptamer complexes as mPF4 tends to form spontaneous complexes in buffers with low
salt concentrations (unpublished observations). Antibody binding was always inhibited by addition of high concentrations of heparin.
Discussion

In this study, we demonstrate a charge- and structure-related interaction between PF4 and nucleic acids and characterize the functional properties of these multimolecular complexes. Nucleic acids induced structural changes in the PF4 molecule similar to those typically observed in PF4/heparin complexes. These structural changes resulted in the exposure of neoepitopes that were recognized by a subset of platelet-activating anti-PF4/heparin antibodies. Moreover, PF4/aptamer complexes induced antibodies in mice which cross-reacted with PF4/heparin complexes.

It is well known that PF4 forms complexes with polyanions other than heparin, such as dextran sulfate, pentosan polysulfate, polyvinyl sulfonate, hypersulfated chondroitin sulfate, and PI-88 (investigational anti-angiogenic drug). The formation of epitopes on these complexes that are recognized by anti-PF4/heparin antibodies is dependent on critical structural features of the polyanions, such as charge density, length, and charge clustering, such as those that occur on branched polysaccharides. Nucleic acids express charged oxygens (O⁻) of phosphate groups that are spaced about 0.5–0.7 nm apart. These dimensions are similar to the known critical charge density of sulfated polysaccharides, in which sulfates are spaced about 0.5 nm apart along the carbohydrate backbone. Thus, the surface exposed phosphate residues in RNA and DNA compounds most likely serve as binding partners for PF4. In fact, polysaccharides in which sulfate groups were substituted by phosphates were demonstrated to bind to PF4, indicating strong PF4-phosphate interaction. Moreover, the phosphate groups of lipid A as a component of LPS also interact with PF4.

Besides charge density, the size of nucleic acids appears to be critical for enhancing binding of PF4 on the platelet surface. Longer constructs augmented PF4 binding to platelets (Figure-3B), while single nucleotides added at the same concentration did not. Similarly,
disaccharides do not bind to PF4, and the synthetic pentasaccharide, fondaparinux, binds only weakly to PF4.\textsuperscript{32,33}

Although nucleic acids seem to share some similarities with heparins in regard to their polyanion character, the structure-function relationships for interactions between PF4 and nucleic acids appear to be more complicated. Other than heparins, nucleic acids can fold into highly organized structures, e.g., those exhibiting spatial clustering of negative charges. While several RNA- and DNA-constructs formed complexes with PF4, the linear homopolymers 45mer-polyA and 45mer-polyC did not (Figure-3), although they were above the critical length and showed a high charge density. Thus, double-stranded domains within nucleic acids seem to be important for binding to PF4. This is in line with our recent observations that double-stranded hairpin-forming, but not single-stranded, nucleic acid oligomers are also potent activators of the intrinsic coagulation pathway by interacting with the RNA-binding cofactor high molecular weight kininogen.\textsuperscript{34}

Figure-6 depicts a simplified model of the interaction between PF4 and nucleic acids: the negatively-charged phosphate residues along the backbone of double-stranded nucleic acids bind to PF4, just as PF4 binds to the sulfate groups of heparin (Figure-6A-B). In contrast, due to their higher flexibility and the loose spatial arrangement of negative charges, single-stranded nucleic acids are more likely to wrap around one PF4 tetramer instead of bridging several PF4 tetramers (Figure-6C). This is mediated by interactions of their phosphate groups with positively-charged amino acid residues of PF4, as it has already been described for polysaccharides comprising >34 sugar units.\textsuperscript{35}

However, the molecular interactions are likely more complex and certain conformations of nucleic acids appear to be more prone than others to form multimolecular complexes with PF4. One example is the reactivity of the 44mer DNA protein C aptamer versus the 77mer RNA FMN aptamer. Although the latter is much longer and has more
double-stranded segments (Figure-1A), anti-PF4/heparin antibodies bound somewhat stronger to PF4/44mer Protein C aptamer complexes (Figure-5D).

Our data raise further questions beyond the scope of the current study. It remains unclear why only about 60% of the anti-PF4/heparin antibodies which induced platelet activation in the presence of heparin also caused platelet activation in the presence of nucleic acids. Even more puzzling is our observation that those sera that induced platelet activation in the presence of nucleic acids did so with most nucleic acids, independent of whether these strongly enhanced PF4 binding to the platelet surface or not. In the absence of a three dimensional model of the structural changes of PF4 induced by different nucleic acids, we can only speculate that the nucleic acids may expose different epitopes than heparins do.

Another hypothetical explanation could be based on the recent studies of Sachais et al, showing that the monoclonal antibody KKO, but not another anti-PF4 monoclonal antibody, is able to induce oligomerization of PF4 also in the absence of heparin, suggesting that some anti-PF4/heparin antibodies are capable of promoting further formation of their own antigen. Following this concept, sera causing platelet activation in the presence of nucleic acids might contain antibodies which—like KKO—can crosslink PF4 molecules. In this case, the negative charge of nucleic acids would initiate antigen formation by allowing close approximation of PF4 molecules which would be sufficient for further oligomerization of PF4 by such anti-PF4/heparin antibodies. Unfortunately, we cannot readily test these hypotheses experimentally due to the polyclonal nature of human antibodies.

Despite these unresolved issues, our study provides an explanation for why patients with major tissue damage develop increased levels of anti-PF4/heparin antibodies compared to patients with minor tissue damage. Increased concentrations of cell-free plasma DNA (1-2µg/mL) have been found in various clinical conditions, including trauma, cancer, and infections, correlating with the severity of trauma or infection. However, due to their
short plasma half-life elevated RNA/DNA-plasma levels return to baseline within a few hours after tissue damage. Yet this might be sufficient to induce an immune response, as also single and minimal applications of heparin have been shown to be sufficient for induction of HIT.42 Cellular RNA and DNA released by the tissue trauma might complex with PF4, potentially enhancing the immune response against PF4/heparin complexes. In vivo formation of PF4/nucleic acid complexes may also explain the formation of anti-PF4/heparin antibodies in patients after major orthopedic surgery even in the absence of heparin,43-45 as well as the relatively high frequency of antibodies in critically ill patients.46 In addition, formation of PF4/nucleic acid complexes might be another explanation for the reported cases of “spontaneous HIT” because all patients who suffered from a HIT-like thromboembolic disorder despite no previous heparin administration, suffered from infections45 or had undergone major orthopedic surgery44.

Finally, our findings might be of relevance for the recognition of unwanted effects of therapeutic oligonucleotides such as aptamers or antisense-RNA. The PF4/44mer protein C aptamer complexes induced a strong immune response in mice, and these complexes are recognized by anti-PF4/heparin antibodies. The resulting antibodies cross-reacted with PF4/heparin complexes, indicating that therapeutic aptamers (with a long plasma half-life) could potentially induce an immune response towards PF4/polyanion complexes. To date, there are no reported cases of HIT-like adverse effects under treatment with therapeutic aptamers and we are not aware on thrombotic complications in the ongoing trials using aptamers. Yet, clinical data are still sparse as the only approved therapeutic aptamer pegaptanib, a RNA aptamer targeting vascular endothelial growth factor (VEGF), is administered intravitreously and systemic concentrations are very low.47 Moreover, thromboembolic complications occurring under pegaptanib, are considered to be a class-effect of anti-VEGF-drugs.48 Beyond this, it should be considered that heparin is much more
immunogenic when given to patients with major proinflammatory states, such as following surgery\textsuperscript{49} or major trauma\textsuperscript{38}. Thus, studies of aptamer administration in healthy volunteers could be expected to underestimate the anti-PF4/aptamer immune response that could occur in patients. The evaluation of the anti-PF4/polyanion immune response within clinical studies is a far more sensitive marker of evaluating the potential of a given aptamer for inducing serious adverse effects than is monitoring for thrombocytopenia and thrombosis.

While induction of an immune reaction resembling typical HIT may be rather unlikely due to the usually low plasma concentrations of aptamers, a strong antibody response to PF4/aptamer complexes may result in an immune reaction resembling “delayed onset HIT”,\textsuperscript{50} an autoimmune-like syndrome in which strongly reactive anti-PF4/polyanion antibodies activate platelets and induce thrombosis even in the absence of heparin. Yet various aptamers differ considerably in their structure and are often chemically modified, which may interfere with their binding to PF4. Using the methods described in this study, it should be possible to screen aptamers for their potential interaction with PF4. We suggest that clinical trials of aptamers or other therapeutic nucleic acid constructs should include screening for the development of anti-PF4/aptamer antibodies and potential clinical features of HIT as part of their safety endpoints.
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Authorship

Miriam Jaax designed and performed the in vitro experiments and wrote the manuscript.

Krystin Krauel designed the study, supervised the experiments, reviewed and interpreted the results and revised the manuscript.

Birgitt Fürll performed and supervised the mouse experiments and interpreted the results.

Thomas Marshall and Bettina Appel designed and synthesized the nucleic acid constructs and reviewed the results.

Sven Brandt, Stephan Block and Christiane Helm performed the CD spectroscopy experiments, reviewed and interpreted the results.

Silvia Fischer and Julia Gansler isolated the cellular RNA, performed the preliminary experiments on PF4-RNA interaction and reviewed the manuscript.

Sabine Müller designed the nucleic acid constructs, reviewed the results and the manuscript.

Klaus T. Preissner created the hypothesis of PF4-nucleic acid interaction, designed the experiments on RNA and wrote the manuscript.

Andreas Greinacher developed the hypothesis of PF4-aptamer interaction, designed the experiments, reviewed the results and wrote the manuscript.

All authors reviewed and approved the final version of the manuscript.

Conflict-of-interest disclosure

None of the authors has to declare any conflict of interest.
References


Legends

Figure 1: Structures of aptamers, DNA and RNA constructs, and nucleotides.
Indicated compounds used in this study are: 1 chemically synthesized by a commercial supplier (PURIMEX, Grebenstein, Germany), 2 enzymatically transcribed in vitro from oligonucleotide templates using T7 RNA polymerase, 3 chemically synthesized in house using the phosphoramidite method (Gene Assembler Special, Pharmacia Biotech, Freiburg, Germany), 4 isolated from MVSMC using an extraction kit (Sigma, Munich, Germany) or the DNazol reagent (Invitrogen, Groningen, Netherlands), 5 purchased from Fermentas (St. Leon-Rot, Germany).

Figure 2: Binding of biotinylated RNA to PF4 and the influence of competitors.
Binding of increasing doses of biotinylated RNA to surface-coated PF4 was performed in the absence (■) or presence of excess DNA (▲), non-biotinylated RNA (□) or heparin (◆). Values represent mean ± SD of 3 independent experiments.

Figure 3: Influence of differently structured nucleic acid compounds on PF4 binding to platelets (for details of the nucleic acid constructs please compare with Figure 1 B-F).
Enhancement of PF4 binding is expressed as x-fold increase compared to PF4 binding to platelets in buffer only. Heparin (grey line) was used as the positive control.
A) PF4 binding to platelets was performed in the presence of increasing doses of cellular RNA (■). Pretreatment with RNase A (□) significantly reduced PF4 binding to platelets (P=.0037).
B) PF4 binding to platelets was evaluated in the presence of increasing doses of nucleic acids with different lengths: 45mer-double stem-loop RNA II (▲), 21mer-hairpin RNA (○), 10mer-single-stranded DNA (◆).
C) PF4 binding to platelets was performed with increasing doses of 45mer-nucleic acid compounds, comprising different structures: 45mer-double stem-loop RNA I (■), 45mer-double stem-loop DNA (△), 45mer-polyA (●), 45mer-polyC (◆), single nucleotides (□).

D) PF4 binding to platelets was measured in the presence of increasing doses of double-stranded vs. single-stranded 21mer-DNA compounds: 21mer-double-stranded DNA (■), 21mer-hairpin DNA (●), 21mer-single-stranded DNA (□). Enhancement of PF4 binding is expressed as x-fold increase of PF4 binding compared to platelets in buffer only. Negative values indicate detachment of PF4 from the platelet surface below the baseline value. All data represent mean ± SD of 3 independent experiments. *** P<.001.

**Figure 4: Influence of aptamers on PF4 binding to platelets (for details of aptamer structure, please compare with Figure 1A).**

The following four aptamers (■) were tested in increasing doses for promoting PF4 binding to platelets in comparison to heparin (grey line) as positive control:

A) 57mer-RNA tetracycline aptamer,

B) 77mer-RNA FMN aptamer,

C) 44mer-DNA protein C aptamer

D) 15mer-DNA thrombin aptamer.

All data represent mean ± SD of 3 independent experiments. The figures indicate that PF4 binding to platelets correlates with the length and the size of double-stranded domains of the aptamers.

**Figure 5A: Structural changes in PF4 induced by the 44mer-DNA protein C aptamer.**

Changes in the circular dichroism pattern of PF4 (20µg/mL) are shown in the absence (n=4, black line) or presence of either heparin (n=3, 6.9µg/mL,◆), or 44mer-DNA protein C
aptamer at different doses (n=2 each, 2.5µg/mL (□); 5µg/mL (Δ); 20µg/mL (○)). Note that the changes in PF4-spectra are rather similar, regardless whether heparin or the aptamer was used. All data represent mean ± SD of n independent experiments. Each experiment consists of at least 5 measurements.

**Figure 5B and C: Human anti-PF4/heparin antibodies induce platelet activation in the presence of nucleic acids and aptamers.**

Mean lag-time until aggregation of donor platelets (at least n=15 for each polyanion) (B) and reactivity of donor platelets expressed as percentage of all tested donor platelets (C) was analyzed in the presence of different anti-PF4/heparin antibody containing sera (at least 4 for each polyanion) and either reviparin (0.2µg/mL; bar 1), cellular RNA (0.5µg/mL; bar 2), 21mer-double-stranded DNA (0.5µg/mL; bar 3), 21mer-hairpin DNA (0.5µg/mL, bar 4), deoxynucleotide-triphosphates (0.5µg/mL; bar 5), 15mer-DNA thrombin aptamer (20µg/mL, bar 6), 44mer-DNA protein C aptamer (8µg/mL, bar 7), 57mer-RNA tetracycline aptamer (5µg/mL, bar 8), or 77mer-RNA FMN-ribozyme (2.5µg/mL, bar 9). Except for the short 15mer-DNA thrombin aptamer, which induces platelet aggregation after 19.0 ± 0.67 minutes and only in 66.6% of all donors, lag-time and reactivity of platelets show only minor differences between the constructs.

**Figure 5D: Binding of anti-PF4/heparin antibodies to PF4/aptamer complexes.**

Anti-PF4/heparin antibodies bind to complexes generated with 20µg/mL PF4 and either 44mer-DNA protein C aptamer (20µg/mL, n=9), 57mer-RNA tetracycline aptamer (10µg/mL, n=4), 77mer-RNA FMN aptamer (10µg/mL, n=4), or heparin (3.3µg/mL, n=9, black bars), while no binding to complexes of PF4 and the 15mer-DNA thrombin aptamer (40µg/mL,
n=4) occurred. Grey bars show inhibition of binding by high heparin (660µg/mL). All data represent mean ± SD of at least 4 independent experiments.

**Figure 5E: Immune response to PF4/aptamer complexes in mice.**
Mice were immunized with either mPF4/44mer-DNA protein C aptamer complexes (n=5), mPF4 alone (n=6), or 44mer-DNA protein C aptamer alone (n=6), and binding of antibodies from the respective sera to mPF4/44mer-DNA protein C aptamer complexes as well as mPF4/heparin complexes was assessed by enzyme-immunoassay in the absence (●) or presence (◇) of 660µg/mL heparin. Median values are marked by black lines.

**Figure 6: Schematic model of complex formation between PF4 and nucleic acids.**
A) The basic amino acid residues (+) in the PF4-tetramer interact with the negatively-charged phosphate groups (-) in double-stranded nucleic acids, thereby forming large multimolecular complexes. B) PF4 binds to the negatively-charged sulfate groups (-) on both sides of the heparin molecule, resulting in the formation of large multimolecular complexes. C) Due to their high flexibility, single-stranded nucleic acids potentially wrap around PF4-tetramers, thereby inhibiting the formation of large, multimolecular complexes with PF4.
Figure 1

A Aptamers

5'-G-T-T-G-G-T-G-T-G-T-G-T-G-G-3'

15mer-DNA thrombin aptamer

5'-G-C-T-C-T-T-A-G-G-3'

44mer-DNA protein C aptamer

5'-G-C-3'

57mer-RNA tetracycline aptamer

77mer-RNA FMN aptamer

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**B** Non – and minimally structured constructs

10mer-single-stranded DNA  

21mer-single-stranded DNA  

45mer-double stem-loop DNA

45mer-double stem-loop RNA I

**C** Complex structured constructs

21mer-hairpin DNA

21mer-hairpin RNA

45mer-double stem-loop RNA II
D Linear double-stranded


21mer-double-stranded DNA

E Linear homopolymer constructs


45mer-polyC

45mer-polyA

F Others

cellular RNA

Nucleotides (Deoxyribonucleotide-triphosphates: ATP, CTP, GTP, TTP)
Figure 3A

Figure 3B
Figure 5D

![Graph showing absorbance (450 nm) for different aptamers and control substances.]

Figure 5E

![Graph showing absorbance (450 nm) for different test scenarios with mPF4 and mPF4//aptamer complex.]
Complex formation with nucleic acids and aptamers alters antigenic properties of platelet factor 4

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