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Platelet activation by charged ligands and nanoparticles: platelet glycoprotein receptors as pattern recognition receptors

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Abstract

Charge interactions play a critical role in the activation of the innate immune system by damage- and pathogen-associated molecular pattern receptors. The ability of these receptors to recognize a wide spectrum of ligands through a common mechanism is critical in host defense. In this article, we argue that platelet glycoprotein receptors that signal through conserved tyrosine-based motifs function as pattern recognition receptors (PRRs) for charged endogenous and exogenous ligands, including sulfated polysaccharides, charged proteins and nanoparticles. This is exemplified by GPVI, CLEC-2 and PEAR1 which are activated by a wide spectrum of endogenous and exogenous ligands, including diesel exhaust particles, sulfated polysaccharides and charged surfaces. We propose that this mechanism has evolved to drive rapid activation of platelets at sites of injury, but that under some conditions it can drive occlusive thrombosis, for example, when blood comes into contact with infectious agents or toxins. In this Opinion Article, we discuss mechanisms behind charge-mediated platelet activation and opportunities for designing nanoparticles and related agents such as dendrimers as novel antithrombotics.

Keywords

CLEC-2; GPVI; nanoparticles; pattern recognition receptors; PEAR1; platelets

History

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Introduction

Platelets are activated by a miscellaneous variety of charged ligands of distinct structures and sizes, ranging from polymers of sulfated polysaccharides to diesel exhaust particles (DEPs).[1] This includes synthetic nanoparticles which activate platelets in proportion to their surface area.[2] Both positively and negatively charged ligands have been shown to activate platelets, with activation mediated through glycoprotein receptors which signal through tyrosine-based signaling motifs in their cytosolic tails or associated membrane proteins. Example receptors include glycoprotein (GP)VI and C-type lectin-like receptor 2 (CLEC-2), which signal through Src family kinases (SFK) and Syk tyrosine kinases, and platelet endothelial aggregation receptor (PEAR)1, which signals through SFKs and PI 3-kinases. Crosslinking of these receptors leads to phosphorylation of tyrosine-based motifs in their cytosolic tail and activation of downstream signaling cascades (see visual abstract).[3]

In this article, we describe the miscellaneous range of charged agonists that stimulate platelet activation through tyrosine kinase receptors, with a special focus on nanoparticles, and how this is influenced by size and surface charge. We speculate that modifications in nanoparticle design could lead to the generation of a new class of antiplatelet agent that prevents charge-mediated receptor clustering and platelet activation.

Nanoparticles in Biomedicine

Nanoparticles are small particles with dimensions under 100 nm.[4] They can have a range of charges and can be comprised of metallic and nonmetallic constituents. Nanoparticles can be engineered for different biomedical applications and have been exploited in several areas, including drug delivery, biosensing and medical imaging.

Metallic nanoparticles, including gold, iron and platinum, have been investigated in biomedical applications both *in vitro* and *in vivo*.[5–8] Gold nanoparticles of varying size under 100 nm have been used for drug delivery, especially for tumor targeting.[9] Gold nanoparticle conjugation to methotrexate (giving ~14 nm nanoparticles) induces cytotoxicity of tumor cells *in-vitro*.[10] Surface area and charge are important factors for gold nanoparticle function and cellular uptake for tumor targeting. Gold nanoparticles (13 nm) also have biosensor utilities and can measure thrombin generation through conjugation to thrombin-binding aptamers.[11] Magnetite, an iron-nanoparticle derivative,

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entrapped with thrombin has been proposed as a novel hemostatic agent. The iron-based nanoparticles can be guided to areas of bleeding by a magnetic field and coagulation is accelerated through fibrinogen injection, shortening hemostasis time by a factor of 6.5.[12] Superparamagnetic iron oxide nanoparticles (SPIONs) are utilized as MRI contrast agents for visualizing liver/spleen tumors and atherosclerotic plaques.[13,14] Platinum nanoparticles have applications in glucose biosensing,[15,16] bioimaging of tumor cells[17] and in mimicking natural enzymes in therapies for oxidative stress.[18]

Nonmetallic nanoparticles, such as carbon nanotubes (CNTs), polystyrene, silica and polyamidoamine (PAMAM) dendrimers, have also been utilized in bioimaging and drug delivery.[19–22] CNTs have been developed as anticancer drugs to help combat multidrug resistance[23] and antigen carriers to help in tumor antigen recognition.[24] Polystyrene nanoparticles (30 nm) have been functionalized to induce cell death of liver-derived tumor cells *in vitro*[21]. Silica nanoparticles are being engineered as potential antigen carriers in vaccine development.[25,26] Dendrimers have utilities in bioimaging and in drug and gene delivery.[22,27,28] They are constituted of branched repeating units, such as polymers, giving rise to a more uniformed nanostructure. Dendrimers can entrap or be conjugated to high molecular weight molecules and become hyperbranched, which are attractive characteristics for drug delivery.[29]

There are a number of important considerations however with the emerging use of nanoparticles in biomedicine that require further attention. The *in vivo* plasma concentrations, half-life and clearance mechanisms of nanoparticles need to be fully characterized in addition to understanding off-target effects, such as platelet activation. The charged nanoparticles, by their very nature, will bind to plasma proteins and to proteins on cells surfaces thus effectively lowering their concentration, and potentially causing off-target effects.

Nanoparticles in Emissions

Despite their utilities in biomedicine, some nanoparticles pose a risk to health. This is illustrated by the increase in incidence of cardiovascular and other diseases in areas with high levels of air-born pollutants[30–32] and with particulate matter in smoke.[33] DEPs (20–70 nm) are regarded as prominent sources of airborne particulate matter.[30] Several epidemiological studies have suggested both short-term and long-term exposure to DEPs and other particulate matter are associated with cardiovascular disease, including venous thromboembolism.[32] Controlled DEP exposure studies have been performed in human subjects, with typical 1–2 h exposure doses ranging for 100–300 μg particulate matter/m^3, with most showing no major effects on inflammatory markers but reduced vasomotor function[34] and adverse vascular endothelial effects.[35] Studies of direct lung translocation of particular matter and *in vivo* effects are mainly assessed in animal models, with several mechanisms of disease pathogenesis proposed. In animal models, nanoparticle exposure has been linked to increased inflammation and platelet activation. In mice, gold nanoparticles have been found in blood, urine and the liver of $ApoE^{-/-}$ mice after inhalation, with a predominance of smaller nanoparticles (<10 nm).[36] The inhaled gold particles accumulate in the vascular lesions of the mice and are associated with mild pulmonary inflammation.[36] In rats, ultrafine carbon nanoparticles can translocate into the circulation following inhalation, with significant accumulation in the liver after 18 h.[37] DEPs were deposited in the distal airways of mice following intratracheal installation and are associated with enhanced collagen-induced platelet aggregation.[38] Different DEP fractions also can induce hypercoagulable states after installation.[39] In rats, intratracheal instillation of DEPs (500 μg/rat) accelerated thrombosis, through reducing occlusion time in a ferric chloride thrombosis model *in vivo*.[40] However, it is important to consider how representable acute, high dose DEP installation in animals is in modeling long-term diesel particle exposure and whether DEP translocation and concentrations in blood would equate to levels observed in human settings, bearing in mind the high level of binding to plasma proteins.

Nanoparticle-mediated Platelet Activation

Several studies have reported that both metallic and nonmetallic nanoparticles induce aggregation and secretion in platelets (Tables 1 and 2). However, most of these studies have been performed in washed platelets, where plasma binding is disregarded, and rarely speculate on the free blood plasma concentration. Interestingly, not only can plasma proteins negate the charge on particles, some such as albumin can form a protein corona around the nanoparticle,[56,57] which may impede nanoparticle-mediated platelet activation but enhance other *in vivo* effects.[52] Various mechanisms have been proposed to influence platelet activation by nanoparticles including size, functional group and charge as discussed below:

Influence of Nanoparticle Size

Differentially sized nanoparticles[2,46,52,58] and different nanoparticle types, both metallic (Table 1) and nonmetallic (Table 2), have been shown to stimulate aggregation of washed platelets and some in platelet-rich-plasma (PRP). There is some evidence that smaller nanoparticles have a greater potency.[29,41,52] For example, an increase in expression of platelet CD62P (*P*-selectin) was reported after exposure to 20 nm gold nanoparticles compared to 70 nm.[41] Potential explanations for this increased potency include the ability of small nanoparticles to reach charged residues close to the membrane or potentially the ease of transport of small nanoparticles into the open canicular system.[41] The former may be relevant for example to the activation of PEAR1 by sulfated polysaccharides from seaweed known as fucoidans. The binding of fucoidan to PEAR1 has been mapped to the heparin-binding domain in the 13th of the 15 EGF repeats in PEAR1, which lies close to the cell surface.[59] Small nanoparticles could potentially mimic this charge effect in fucoidans and thus activate PEAR1.

Influence of Nanoparticle Surface Area

We have recently shown that platelet activation is proportional to nanoparticle surface area and revealed that surface area is a critical factor for mediating platelet activation.[2] There is an inverse surface area ratio relationship, whereby smaller nanoparticles have larger surface areas. The degree of aggregation in proportion to the surface area and shows a bell-shaped relationship. The bell-shaped curve is consistent with activation being mediated by receptor clustering, with increased concentrations of nanoparticles competing with each other for receptor binding. Alternatively, the bell-shaped curve could be due to charge or steric effects preventing aggregation. It is important to note that the nanoparticles are much greater in size than the majority of platelet receptors. For example, the theoretical maximum height

Table 1. **Platelet activation by metallic nanoparticles**. Summary of metallic nanoparticles that have been shown to activate platelets (through aggregation, microscopy or flow cytometry studies) and the proposed mechanism behind the nanoparticle function. WP = studies performed using a form of washed platelets (no plasma proteins). PRP = studies performed using platelet-rich plasma (PRP; containing plasma proteins).

Nanoparticle	Size (nm)	Shape	Charge	Concentration	WP/ PRP	Function	Proposed Mechanism(s)	Ref
Platinum	7 to 73	Spheres	Negative	$\log_{10}$ 1–4 cm^2 /mL	WP	Activatory	Surface area and tyrosine kinases	2
Gold	18, 55, 68	Spheres	Negative	20–40 μM	WP	Activatory	Tyrosine kinases	41
Gold	30 to 50	Spheres	Negative	0.42–0.45 mg/mL	PRP	No effect	-	42
Gold	5 to 30	Spheres	Negative	5–40 μM	PRP	No effect	-	43
	60	Spheres	Negative	5–40 μM	PRP	Inhibitory	Size and surface ligand	
Gold	12	Spheres	Negative	1.2 nM	PRP	No effect	-	44
	28–85	Rods	Negative	1.2 nM	PRP	No effect	-	
Silver – PVP/citrate	20	Spheres	Negative	1–80 μg/mL	PRP	No effect	-	45
Silver	10–100	Spheres	Negative	50–250 μg/mL	WP	Activatory	GPIIb/IIIa /calcium release	46
Silver	13	Spheres	Positive	50 μM	WP	Inhibitory	-	47
Silver	16	Spheres	Positive	5 μg/mL	PRP	No effect	-	48
Iron oxide – starch	45	Oval	Neutral	160 μM	PRP/WP	No effect	-	49
Iron oxide – citrate	35	Spheres	-	160 μM	PRP/WP	Inhibitory	Charge	
Iron oxide – 5PAA/5 HAA	5–6	Spheres	Negative	8–1000 μg/mL	PRP	Activatory	Size and functional group	50
Iron oxide – 5HA	5–6	Spheres	Negative	8–1000 μg/mL		No effect	-	
Iron oxide – 5CS	5–6	Spheres	Positive	8–1000 μg/mL		No effect		
Iron oxide – 10PAA	10	Spheres	Negative	8–1000 μg/mL		Activatory	Size and functional group	
Iron oxide – 30PAA	30	Spheres	Negative	8–1000 μg/mL		No effect	-	

Table 2. **Platelet activation by nonmetallic nanoparticles**. Summary of nonmetallic nanoparticles that have been shown to activate platelets (through aggregation, microscopy or flow cytometry studies) and the proposed mechanism behind the nanoparticle function. WP = studies performed using a form of washed platelets (no plasma proteins). PRP = studies performed using platelet-rich plasma (PRP; containing plasma proteins).

Nanoparticle	Size (nm)	Shape	Charge	Concentration	WP/ PRP	Function	Proposed Mechanism(s)	Ref
Polystyrene latex – amine	60	Spheres	Positive	12.5–100 μg/mL	PRP	Activatory	Functional group	51
Polystyrene latex – carboxyl/ unmodified	60	Spheres	Negative	12.5–100 μg/mL	PRP	No effect	-	
Polystyrene latex – amine	50, 100	Spheres	Positive	8–60 μg/mL	WP/ PRP	Activatory	Size and charge	52
Polystyrene latex – carboxyl/ unmodified	50, 100	Spheres	Negative	8–60 μg/mL	WP/ PRP	Activatory	Size and charge	
Polystyrene	25–201	Spheres	Negative	$\log_{10}$ 1–3 cm^2/ mL	WP	Activatory	Surface area and charge	2
Diesel exhaust particles	40–70	Agglomerates	-	0.2–12 μg/mL	WP	Activatory	Receptor-dependent signaling	38
Diesel exhaust particles	4 − 35	-	-	5–50 μg/mL	WP	Activatory	Charge Tyrosine kinases	1
Single/multi- walled carbon nanotubes	-	Tubes	-	0.2–300 μg/mL	WP	Activatory	Receptor-dependent signaling	53
Single walled carbon nanotubes	<2, 1–2	Tubes	-	100 μg/mL	PRP	Activatory	Calcium influx	54
Multi walled carbon nanotubes	30, 60–100	Tubes	-	10–500 μg/mL		Activatory	Calcium influx	
Multi walled carbon nanotubes	1–2	Tubes	-	25–200 μg/mL	WP	Activatory	Receptor-dependent signaling	55
PAMAM G4 – G6 amine-terminated	4.2–7.5	Spheres	Positive	1.6–100 μg/mL	PRP	Activatory	Functional group/charge	29
PAMAM G3 – G6 carboxyl-terminated	3.5–7.6	Spheres	Negative	1.6–100 μg/mL		No effect	-	
PAMAM G3 – G6 hydroxy-terminated	3–6.5	Spheres	Neutral	1.6–100 μg/mL		No effect	-	

of CLEC-2 above the membrane is ≈12 nm, which is similar to that of the smaller nanoparticles (Supplementary Figure 1).

Influence of Nanoparticle Charge

Platelet activation *in vitro* has been shown following exposure to both negatively and positively charged metallic[47,48] and nonmetallic[29,51,52] nanoparticles. Platelet function is not altered with exposure to nanoparticles with neutral charge.[29] The functional group attached to the nanoparticles could potentially be involved in nanoparticle–platelet interactions. Different carboxyl groups have been shown to affect nanoparticle potency for platelet activation.[52] Amine modifications of nanoparticles, giving positive charges, can increase platelet aggregation.[51] Large (G4-G6) cationic PAMAM dendrimers associated with increased numbers of amine groups are able to induced platelet aggregation compared to the other formulations of neutral and anionic PAMAM dendrimers.[29] Negatively charged platinum,[2] silver,[46] gold,[41] polystyrene[2] and iron[50] nanoparticles all activate platelets with tyrosine kinase dependent or rapid signaling-dependent mechanisms, leading to Ca^{2+} release (Tables 1 and 2). Tailoring nanoparticles to have positive or negative charges will be

a critical approach in determining the mechanism(s) behind charge-mediated platelet activation and in blocking these interactions.

Synthesis of Charged Nanoparticles

Methods for synthesizing nanoparticles include chemical (reduction of metal precursor), physical (laser ablation) or biological (plants, bacteria).[6] Chemical synthesis methods are well-suited for controlling nanoparticle size, functionalisation and monodispersity. A detailed example of chemical synthesis of metallic nanoparticles is shown in Figure 1, whereby small nanoparticles (seeds) act as a template to grow larger nanoparticles.[6,60,61] Nanoparticles can be functionalized by the addition of ligands, surfactants, polymers, biomolecules or thiols during or after synthesis reactions.[62–65] Thiols can be physisorbed onto the nanoparticle surface forming a monolayer surrounding the nanoparticle core and can carry different terminal head groups (negatively/positively or neutral

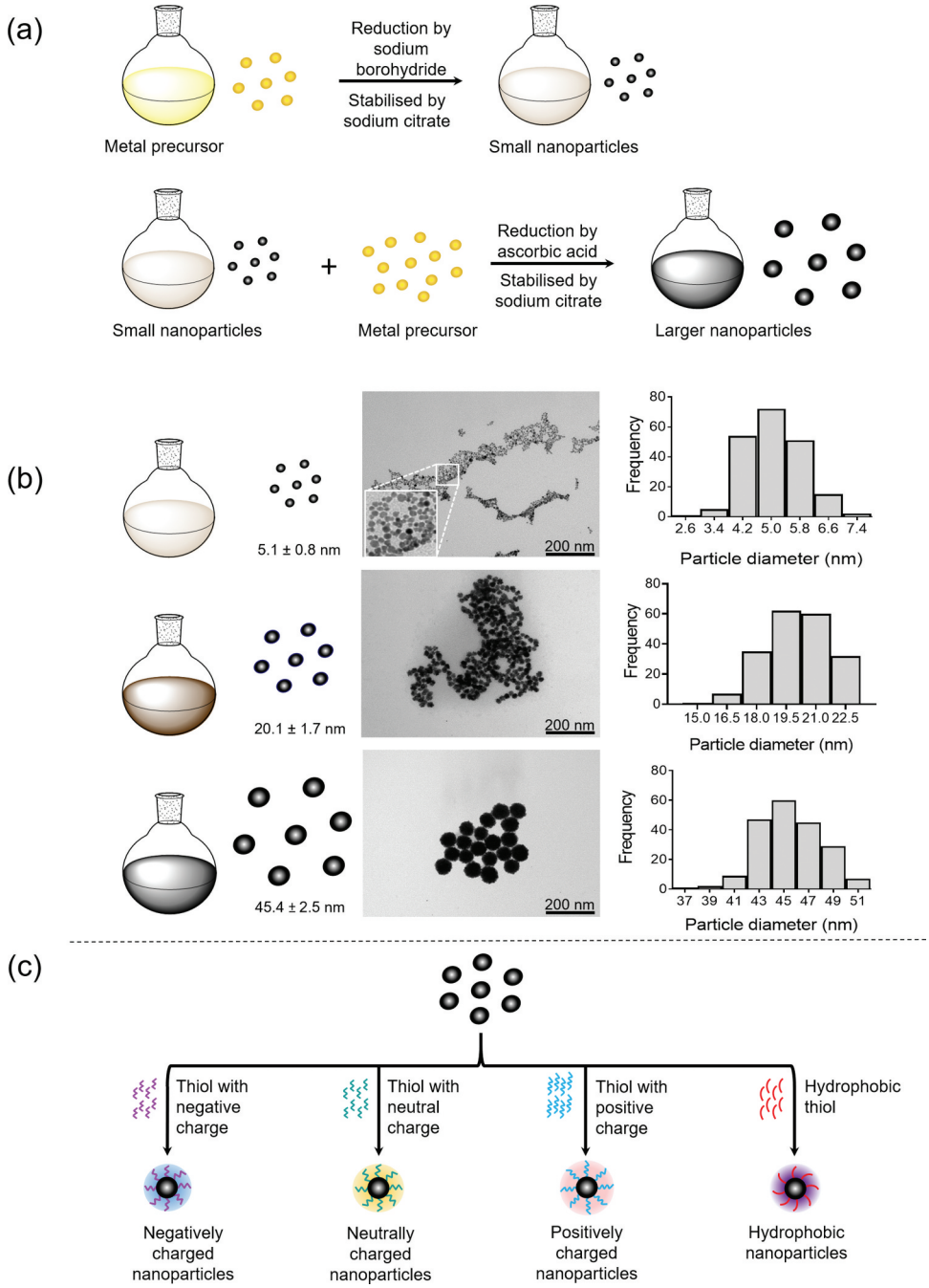

Figure 1. **Nanoparticle synthesis**. a) Metallic nanoparticles (gold/platinum) can be synthesized by the nucleation process, whereby small nanoparticles (seeds) are a template for larger nanoparticle (up to 100 nm) growth. Stable nanoparticles with controlled sizes are achieved by selecting the suitable reducing agents and stabilizing agents in addition to precursor (metal salts) concentrations and temperature. Small nanoparticles are initially synthesized by the reduction of the metal precursor by sodium borohydride and stabilized by sodium citrate. Larger nanoparticles are synthesized using these small nanoparticles with ascorbic acid and sodium citrate. b) Examples of different sized platinum nanoparticles synthesized by the nucleation process. Transmission electron microscopy (TEM) images are employed to obtain the average nanoparticle diameter to generate the distribution curve. c) Functionalisation of nanoparticles by thiols to produced nanoparticle with negative or positive charges (charged = also potentially hydrophilic) and hydrophobic nanoparticles.

charge) and hydrophobic moieties (Figure 1C). Thiol addition to give a specific surface charge could be beneficial for designing nanoparticles for therapeutics.

Mechanism of Charge Mediated Platelet Activation

Ligand engagement of platelet receptors leads to phosphorylation of signaling proteins, increased Ca^{2+} flux and platelet aggregation via enhanced fibrinogen binding to integrin GPIIb/IIIa.[66] Charged nanoparticles can potentially induce receptor activation through homoreceptor or heteroreceptor cross-linking, either through direct receptor crosslinking on individual platelets. They can also potentially induce activation through a conformational change in the receptor or removal of inhibitory pathways as illustrated in Figure 2B and C. Also, they can potentially produce passive agglutination by crosslinking of proteins on adjacent platelets. This is sometimes seen with high concentrations of nanoparticles which induce GPIIb-IIIa independent aggregation. However, lower concentrations favor activation of GPIIb-IIIa in part mediated by the feedback action of secondary agonists such as ADP and thromboxane (TxA_2).

Charge-mediated mechanisms of platelet activation are primarily restricted to glycoprotein receptors as these are activated through clustering in contrast to G protein-coupled receptors which are activated through a conformational change which require exquisite binding of the ligand. Several platelet glycoprotein receptors have been shown to be activated by charged ligands including:

GPVI

GPVI is a member of the immunoglobulin (Ig) receptor superfamily expressed on platelets and megakaryocytes and best known as a receptor for collagen.[67] GPVI is activated by a diverse range of endogenous and exogenous ligands (Table 3), including fibrin and fibrinogen,[68–70] extracellular matrix proteins (laminin, fibronectin, galectin-3[71] and vitronectin[67,72]), positively charged histones[1] and the neuronal proteins, reelin and β-amyloid.[73,74] Negatively charged molecules, such as chondroitin sulfate, heparins and small polyanions block fibrin-mediated aggregation and GPVI shedding, supporting a role for charge-mediated GPVI activation.[75] Furthermore, serglycin, a sulfated proteoglycan contained in platelet α-granules, also appears to be important in charge neutralization and regulation of charge-mediated GPVI shedding, as convulxin-mediated GPVI shedding is increased in serglycin knock-out mice.[76]

A number of proteins ligands for GPVI have regions of negative net charge, including fibronectin, fibrinogen, β-

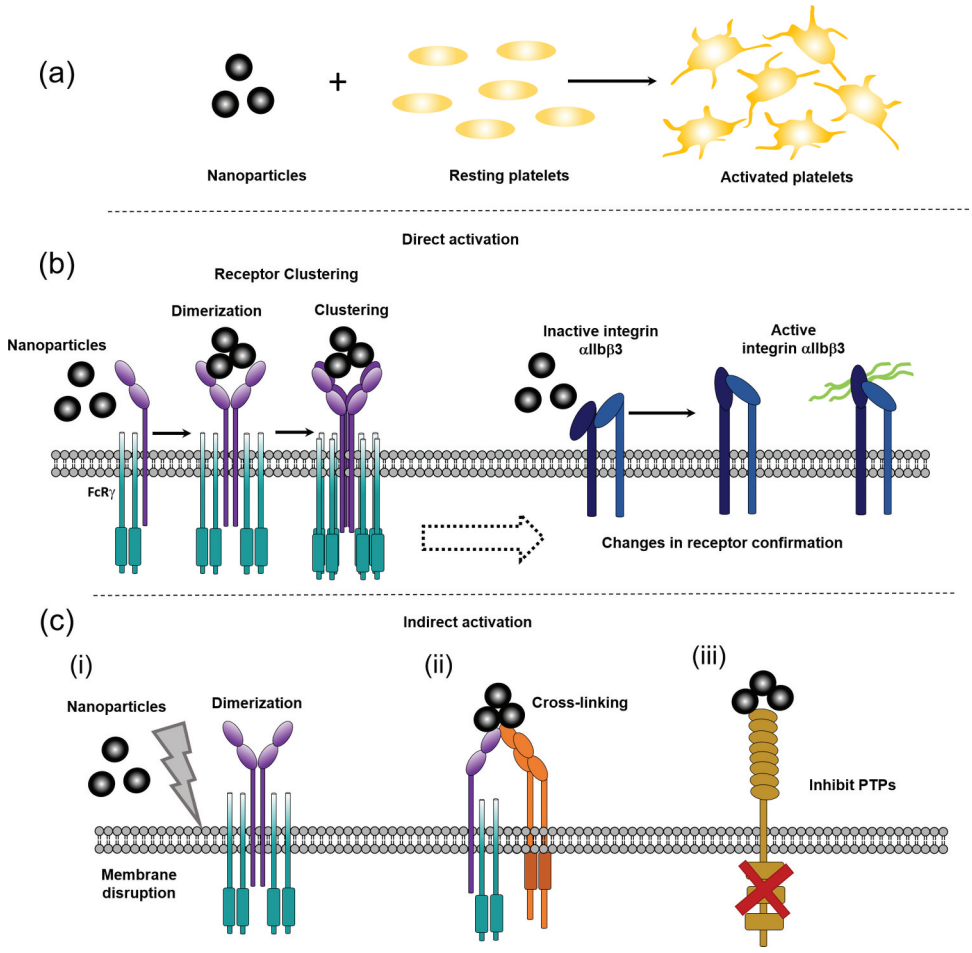

Figure 2. **Schematics of potential mechanisms behind nanoparticle induced platelet activation**. a) Nanoparticles are small particles (<100 nm) that can activate platelets, through different proposed mechanisms. b) Direct nanoparticle driven platelet activation. Nanoparticles could potentially bind directly to platelets, such as GPVI, leading to ITAM signaling, dimerization and clustering or through causing conformational changes in receptors, like integrin αIIbβ3 (GPIIb/IIIa) changes from an inactive confirmation to an active. c) Potential in-direct mechanisms for nanoparticle driven platelet activation. (i) nanoparticles cause general membrane disruption leading to receptor dimerization, (ii) nanoparticles crosslinking different platelet receptors, (iii) nanoparticle binding/activation leads to inhibition of protein-tyrosine phosphatases (PTPs)/ ITIM receptors, promoting platelet activation. Nanoparticles are not to scale, they are represented here are small nanoparticles (<50 nm), larger nanoparticles can be larger than the receptor height above the membrane (see Supplementary Figure 1).

amyloid and phosphorothioate oligonucleotides.[77] In addition, DEP-mediated activation has previously been shown to be abolished in platelets from GPVI-deficient mice.[1] Here, we expanded on this to show negatively charged DEPs (20–70 nm) and negative charged surfaces also activate human platelets through GPVI, using platelets from homozygous patients (Figure 3A), who have a mutation in the *GP6* gene preventing expression[78] (see Supplementary Materials for full patient details). These patients have no GPVI expression as shown by flow cytometry and western blotting, whereas levels of other glycoprotein receptors, including CLEC-2, GPIIb/IIIa and FcγRIIA are within the normal range.[78,79] Stimulation of tyrosine phosphorylation of proteins in the GPVI signaling pathway, including Syk, LAT and PLCγ2, by DEPs is abolished in the patients (Figure 3A). In line with these results, platelets undergoing spreading (i.e. filopodia and lamellipodia formation) on negatively charged glass surfaces in association with an increase in tyrosine phosphorylation including LAT and PLCγ2 (Figure 3B). Spreading and phosphorylation of both proteins are abolished in the presence of the Syk inhibitor PRT-060318 (Figure 3B) consistent with the critical role for GPVI. Tyrosine phosphorylation of Syk and FcRγ-chain, and spreading, is also induced on polystyrene, with phosphorylation of both proteins blocked by PRT-060318 (Supplementary Figure 2). Tyrosine phosphorylation of several other proteins is retained in the presence of the Syk inhibitor on both surfaces demonstrating that other pathways contribute to the increase in phosphorylation but that these do not induce spreading.

GPVI has several areas of charge in its two Ig domains as shown in Figure 4A, and contains a highly negatively charged stalk that is highly *O*-glycosylated and charged due to sialylation.[80,81] Site directed mutagenesis studies[82] along with co-crystallization of GPVI with CRP and a blocking nanobody show critical charged residues (predominantly positively charged) at positions E21, R38, E40, R46, K59, R60, R67 and R166 in the D1 domain.[82–84] The crystallization of GPVI with a nanoparticle or dendrimer could reveal whether these residues or other residues provide a surface on GPVI to mediate binding of charged ligands. This will also help determine whether nanoparticles and other charged ligands bind directly to specific epitopes or in ligand-binding regions on GPVI, or if GPVI activation is mainly driven by the general charge of the nanoparticles/ligands. Furthermore, developing a blocking agent to prevent GPVI-charged ligand engagement could help to disrupt thrombus propagation and reduce vessel occlusion.

CLEC-2

CLEC-2 is a receptor for the highly sialylated membrane protein podoplanin and oxidized heme (hemin).[85] CLEC-2 has also been shown to be activated by DEPs in human and mouse platelets, and by negatively charged poly-sulfated fucoidans in mouse platelets (Table 3).[1,86]

The extracellular domain of CLEC-2 has been co-crystallized with a podoplanin peptide (containing the conserved binding sequence) and the snake venom rhodocytin. The sequence within podoplanin, EDXXXT (single amino acid code), is known as a platelet aggregation-stimulating (PLAG) domain.[85,87] These are conserved in mammals and mediated podoplanin-induced platelet activation. The structure revealed that threonine in the PLAG-3 domain is glycosylated and capped with sialic acid which is critical for binding to CLEC-2. The rhodocytin α-subunit possesses a unique Glu-Asp sequence which is also critical for binding to CLEC-2. The interaction with both ligands is mediated through four arginine residues (R107, R118, R152 and R157) that create a charged surface on CLEC-2 (depicted in Figure 4B). Consecutive acidic residues within the PLAG-3 domain of podoplanin, and the negatively charged residues within the N-terminal loop of the α-subunit of rhodocytin bind through electrostatic contacts to the charged surface within the CLEC-2 C-type lectin-like domain, while additional polar contacts reenforce the binding interface. Ligand binding can be abolished by mutation of the essential arginine residues within CLEC-2 to

Table 3. **Multiple ligands of the tyrosine kinase receptors, GPVI, CLEC-2 and PEAR1**. GPVI, CLEC-2 and PEAR1 are activated by multiple ligands. Top row = endogenous ligands recognized for GPVI, CLEC-2 and PEAR1. Bottom row = exogenous ligands for GPVI, CLEC-2 and PEAR1.

	GPVI	CLEC-2	PEAR1
Endogenous Ligands	Collagen Fibrin Fibrinogen* α_5-Laminin Fibronectin Vitronectin EMMPRIN (CD147) Adiponectin Amyloid Aβ40 Galectin-3 Reelin	Podoplanin Hemin	Immunoglobulin E receptor subunit α (FcεRIα)*** Heparin-based glycosaminoglycans (?)
Exogenous ligands	Charged peptides Collagen-related peptide (CRP) Convulxin and other snake toxins Diesel exhaust particles Histones Phosphorothioate oligonucleotides PAM3CSK4 Sulfated polysaccharides	Rhodocytin Sulfated polysaccharides** (e.g. fucoidan, dextran sulfates) Diesel exhaust particles	Sulfated polysaccharides (e.g. fucoidan, dextran sulfates) Synthetic α-L-fucoside pendant glycopolymers/fucoidan mimetics

***Activates GPVI in human but not mouse platelets**
****Activates CLEC-2 in mouse but not human platelets**
***** Activates PEAR1 in human but not mouse platelets**
(?) – proposed but not confirmed [59].

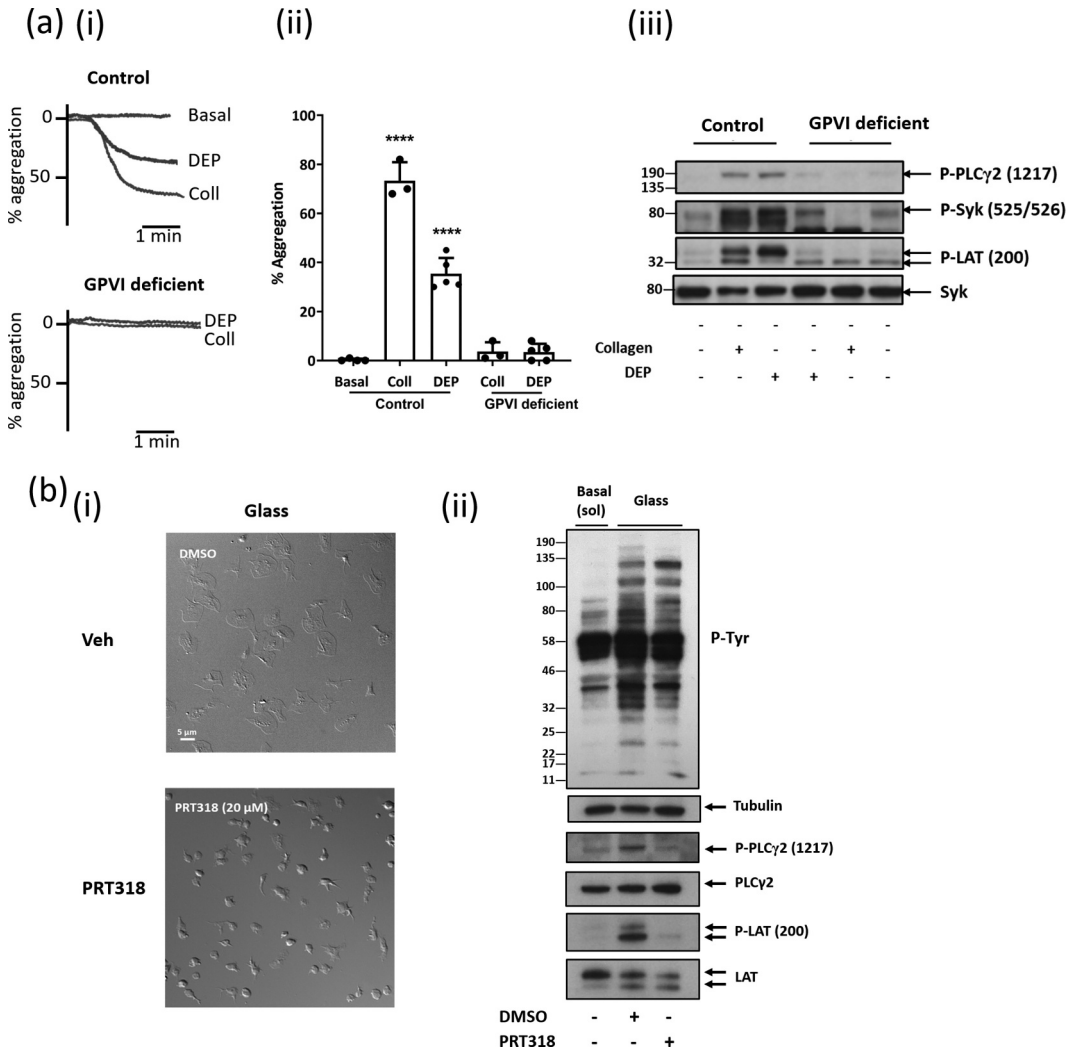

Figure 3. **Charged DEPs and negative charged surfaces activate human platelets through a GPVI and Syk dependent manner**. a) Commercially bought DEP (50 μg/mL; see Supplementary Materials for details) induced platelet aggregation is abolished in GPVI-deficient patients. (i) Representative aggregation trace of DEP and collagen (Coll; 30 μg/mL) induced aggregation in washed platelets ($4x10^8$/mL) from controls ($^{+/+}$) and GPVI deficient patients (individuals homozygous ($^{-/-}$) for a mutation in *GP6* resulting in truncated GPVI). For more details see Supplementary Materials. (ii) Significant reduction in DEP-aggregation and collagen-induced platelet aggregation in GPVI deficient individuals. One-way ANOVA with Tukey's multiple comparisons test, n = 3–5, ****p < .0001 to basal. (iii) Representative western blot for tyrosine phosphorylation of PLCγ2, Syk and LAT following collagen and DEP stimulated washed platelets from GPVI-deficient individuals and control individuals. b) Platelet spreading on glass (charged surface) is abolished with Syk inhibition. (i) Washed platelets ($2x10^7$/mL) spread on glass (negatively charged surface) for 30 min. Platelets were pre-incubated with 20 μM PRT-060318 (Syk inhibitor) or DMSO (vehicle) 5 min before spreading. Scale bar = 5 μm. (ii) Western blot for tyrosine phosphorylation of signaling proteins after platelets were spread on glass. Representative of 3 experiments.

uncharged residues, such as alanine,[87] highlighting the importance of charge during CLEC-2: ligand binding.

PEAR1

Human PEAR1 is a novel platelet and endothelial receptor that mediates powerful activation of platelet by sulfated polysaccharides, including fucoidans and dextran-sulfate.[59] Sulfation is critical for activation. The site of interaction has been mapped to the thirteenth EGF-like repeat in PEAR1 which contains a cluster of positively charged amino acids in a conserved heparin binding-like consensus sequence. The co-crystallization of PEAR1 with its ligands will map the critical amino acids and potentially provide information on the endogenous ligand. Interestingly, we have shown that in mouse platelets, sulfated glycopolymers mediate activation through CLEC-2 with only a partial role for PEAR1[59] and speculate that this may be due to the much higher expression of CLEC-2 in mouse platelets compared to humans.[88] If this is the case, this illustrates the promiscuity of charged ligands consistent with the charge playing a dominant role in mediating activation.

Other Receptors

Other surface glycoproteins are also anticipated to bind to charged ligands and may facilitate activation of platelet glycoprotein receptors. Examples include the role of GPIb in supporting activation of mouse platelets by fucoidans[59] and the observation that adhesion but not spreading of platelets to fibrin and fibrinogen is retained in the absence of GPVI and GPIIb/IIIa.[70] These receptors therefore may act as adhesion receptors which help to increase

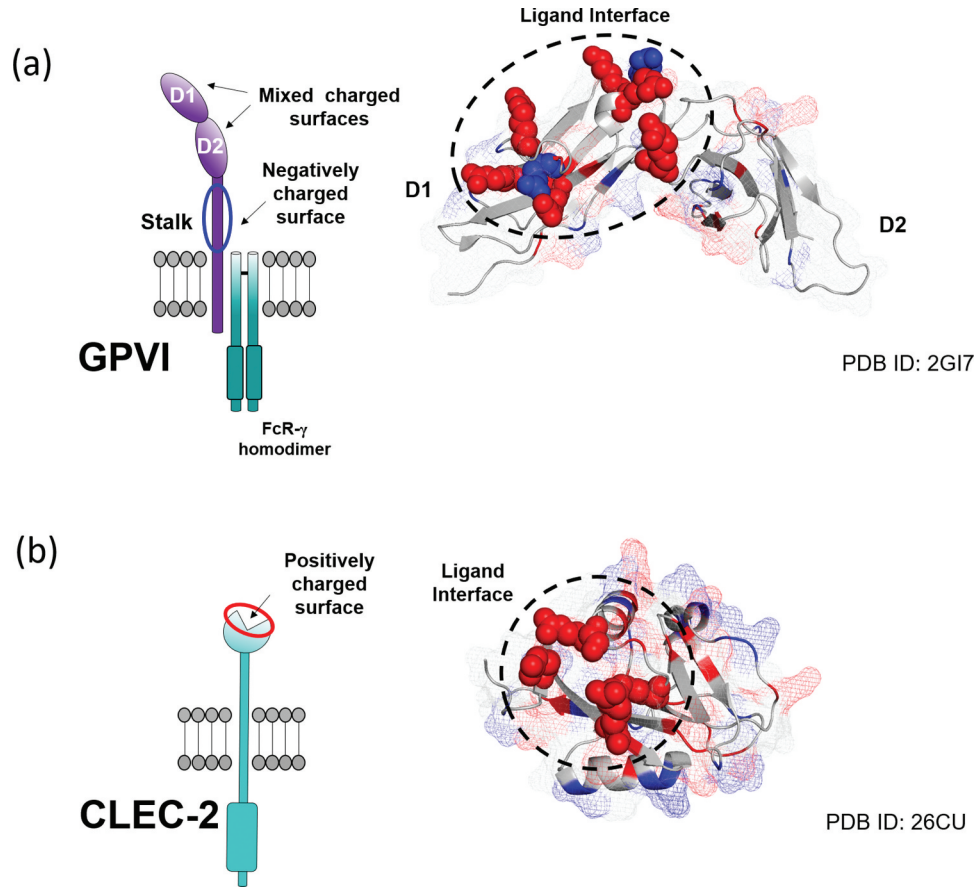

Figure 4. **Charge distribution and ligand-binding interface mapped onto the surface of a) GPVI (PDB: 2GI7) and b) CLEC-2 (PDB: 2C6U) extracellular domains**. Charged residues of the ligand binding interface are represented as spheres with their color corresponding to charge (red: positive, blue: negative). Additional charged residues are highlighted throughout the domains with their surface coverage displayed as a mesh.

binding to the tyrosine kinase receptors for rapid charge-mediated signaling.

Relevance of Charge-mediated Platelet Activation?

Platelets are exposed to a variety of charged matrix proteins in both the blood and following lesion to the vessel wall, including at sites of plaque formation. Atherosclerotic plaques are rich in lipid deposits, connective tissue (collagen, fibronectin), proteoglycans and necrotic cell debris[89,90] and form large areas of charge thereby forming a highly prothrombogenic surface.[91] Many of the components of an atherosclerotic plaque present a charged surface for platelets to adhere, activate and aggregate. Moreover, lesion atherosclerotic plaques mediate activation through GPVI.[92,93]

Charged exogenous ligands induce powerful activation of washed platelets, although not all are active in blood due to binding to plasma proteins, such as albumin.[29] This is the case for nanoparticles which bind predominantly because of their charge, whereas protein ligands such as snake venom toxins form a conformationally constrained interaction with their receptors and retain their ability to activate platelets in blood. This has important implications for the clinical significance of exposure to DEPs, with the link to cardiovascular disease more likely to be mediated through damage to blood endothelial cells.

A special consideration is needed for the exposure of platelets to foreign charged surfaces in dialysis and with use of extracorporeal membrane oxygenation (ECMO) and left ventricular assist devices (LVADs).[94] Hemostatic complications, both bleeding and activation are frequent in the latter[94,95] and associated with build of thrombi on the oxygenation membrane and connectors. It is not known if this is the result of a direct interaction of platelets with the surface, or protein immobilized to the surface and/or to other factors such as coagulation initiation or changes in shear stress.[72,96]

Binding to Microvesicles?

Platelets rapidly shed microvesicles which express the majority of surface receptors other than GPVI, which has shown to be lost as the result of shedding.[97] The microvesicles could potentially therefore interact with charged ligands, however the functional significance of this is uncertain.

Are Platelet Glycoproteins Receptors for DAMPS and PAMPs?

Damage-associated molecular patterns (DAMPs) is the umbrella term for cell debris and soluble mediators released after damage based on the theory of "self" driving immune responses.[98] DAMPs, like pathogen-associated molecular patterns (PAMPs), bind to pattern recognition receptors (PRRs) to induce pro-inflammatory cytokine production and invoke immune responses, which can be associated with poor patient outcome.[99] PRRs can bind multiple DAMPs thereby mediating activation to a wide range of stimuli.

Platelets express many PRRs belonging to multiple families including toll-like receptors (TLRs), NOD-like receptors (NLRs) and C-type lectin-like receptors and are involved in promoting immune responses both in infection and with sterile inflammation.[100] Platelet PRRs recognize a wide range of

DAMPs including extracellular/mitochondrial DNA, histones and high mobility group box protein (HMGB)-1.[101–104] However, most of these receptors are expressed in low level and, for many, platelets lack their downstream signaling components (see below).

Platelets express several TLRs, including surface TLR-1, -2, -4 and -6,[105,106] with TLR-3, -7 and -9 being located in platelet endosomes and translocated to the surface after activation.[100] TLRs signal via recruitment of Toll/Il-1 receptor (TIR) domain containing adaptors, such as MyD88, TIRAP and TRIF (depending on TLR function), resulting in activation of transcription factors NF-kB, IRFs and MAPKs, increasing pro-inflammatory cytokine production.[107] However, platelet lack many of the TLR signaling proteins and lack a nucleus.[106] For example, TLR-4 signaling on other cell types requires CD14, which is not present on platelets.[108] TLR-4 is a receptor for LPS and has been reported to mediate platelet activation,[106,109] but this is controversial with several groups unable to shown activation by LPS.[109–111] It has also been proposed that LPS does not alter TLR-4 expression or enhance platelet–leukocyte interactions,[109] suggesting TLR-4 independent mechanisms. Other DAMPs such as histones and HMGB-1 have been proposed to mediate platelet activation through TLR-2 and TLR-4.[103,104] However, TLR-independent mechanisms cannot be ruled out, with histones activating GPVI signaling proteins[1] and HMGB-1 also activating the receptor for advanced glycation end product (RAGE).[112] In summary, platelets express several Toll receptors but for most of these they lack the signaling proteins to mediate responses such as aggregation, secretion and spreading on surfaces. This means that platelets require other classes of PRRs receptors to mediate their primary functional responses.

CLEC-2 is the most common C-type lectin-like receptor on platelets and is an emerging PPR. Platelet CLEC-2 is involved in invoking immune responses to HIV and cooperates with DC-sign in dengue infections.[113,114] Heme, released from red blood cells following hemolysis, also activates platelets through CLEC-2 signaling, emphasizing the importance of CLEC-2 as a PRR receptor.[115]

As summarized above, GPVI, CLEC-2 and PEAR1 and shown in Table 3 are activated by a wide range of charged ligands suggesting that they should be considered to be PRRs. This could be an evolutionary conserved mechanism to activate platelets at sites of inflammation and vascular damage in a rapid manner. Designing charge agents to disrupt the interaction of the major signaling glycoprotein receptor in platelets with DAMPs could therefore reduce platelet activation in arterial thrombosis and thromboinflammation.

Charge Interactions in Coagulation

Charge interactions are also important in driving coagulation and are thought to present a primitive and evolutionary conserved way to prevent excessive bleeding. FXII contact with negatively charged surfaces on exposed blood vessels or binding of exogenous negatively charged molecules such as polyphosphate, heparin and nucleotide RNA released after damage or in inflammation leads to FXII activation and initiates coagulation through contact activation.[116–119] Potentially, this could work in combination with charged-mediated activation of platelets to prevent excessive blood loss and promote inflammation in times of damage and infection. Tailoring nanoparticles to model important protein–charge interactions with key coagulation factors could provide novel therapeutic angles for charge neutralization in times of hypercoagulation and thrombosis.

Conclusions

There is an urgent need for more powerful antiplatelet therapy that also preserves hemostasis. In this Opinion Article, we argue that this could be achieved by considering several of the key platelet signaling receptors as PRRs that are activated by binding to charged ligands, notably GPVI, CLEC-2 and PEAR1. It is of great interest to map the mechanisms behind charge-mediated platelet activation, and to develop blocking therapeutics. Tailoring nanoparticles and dendrimers to have specific surface areas and charge may provide a valuable tool for disrupting platelet-ligand engagement or neutralize charge related membrane disruptions, providing a new approach for development of novel antithrombotics.

Contributions

S.J.M and P.P. performed the literature review, generated figures and wrote the manuscript. E.M.M and A.S discussed research and generated models. L.G.Q., G.P., M.N., D.M. and S.P.W. performed research experiments. K.C. discussed research and contributed intellect to the review. P.M.M. and S.P.W. led the research, planned the review and wrote the manuscript. All authors contributed, read and edited the manuscript, and approved it for publication.

Disclosure Statement

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