Review

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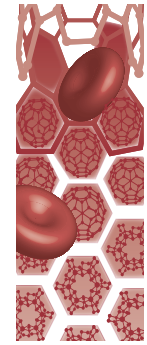
Nanomedicine



Nanodiagnostics, nanopharmacology and nanotoxicology of platelet–vessel wall interactions

In physiological conditions, the interactions between blood platelets and endothelial cells play a major role in vascular reactivity and hemostasis. By contrast, increased platelet activation contributes to the pathogenesis of vascular pathology such as atherosclerosis, thrombosis, diabetes mellitus, hypertension and carcinogenesis. Nanomedicine, including nanodiagnostics and nanotherapeutics is poised to be used in the management of vascular diseases. However, the inherent risk and potential toxicity resultant from the use of nanosized (<100 nm) materials need to be carefully considered. This review, basing on a systematic search of literature provides state-of-the-art and focuses on new discoveries, as well as the potential benefits and threats in the field of nanodiagnostics, nanopharmacology and nanotoxicology of platelet–vessel wall interactions.

Keywords: adhesion • aggregation • endothelial cells • interactions • nanodiagnostics • nanoparticles • nanopharmacology • nanotoxicology • platelet • vessel wall

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In order to review state-of-the-art in interactions between nanomaterials, blood platelets and the vessel wall, we have conducted a systematic search of literature as outlined on Figure 1. The remaining references have been selected by the authors of this review.

Platelets & the vascular endothelium – an introduction

Platelets

Platelets originate from megakaryocytes. They are anucleated, disc-shaped cytoplasmic fragments, approximately 2 μm in size, and a mean volume of 7 μm^3. Platelets have a short life span and circulate in the blood for approximately 10 days [1]. Under physiological conditions and in the absence of any inducing stimuli, platelets circulate in a quiescent state; however, when activated, platelets undergo rapid shape changes (Figures 2 & 3) to support the release of intraplatelet granules and form the primary plug. Platelets contain three types of granules (alpha-granules – forming ~10% of platelet volume, dense granules and lysosomes), each storing different mediators [1,2].

The main physiological role of platelets is to secure primary hemostasis and maintain the integrity of the vascular wall [3]. However, they have also other functions, and are implicated in immunity and inflammation [4], vascular permeability [5] and cancer metastasis [6]. It is well known that platelets contribute to the pathogenesis of vascular dysfunction including atherosclerosis, thrombosis, diabetes mellitus and hypertension, and thus are implicated in cerebrovascular and cardiovascular disorders such as ischemic stroke and myocardial infarction [7].

Under physiological conditions when blood flow is laminar, due to shear stress platelets flow close to endothelial cells (ECs) along the vessel wall and promote vascular integrity [8]. Several studies have shown that shear stress may influence platelet aggregation through different ligands such as the vWF, GPIb/IX, GPIbα and GPIIb/IIIa [1,9].

Both adhesion (platelet–vessel wall interactions) and aggregation (platelet–platelet interactions) of platelets largely depend on the blood flow and shear conditions at sites

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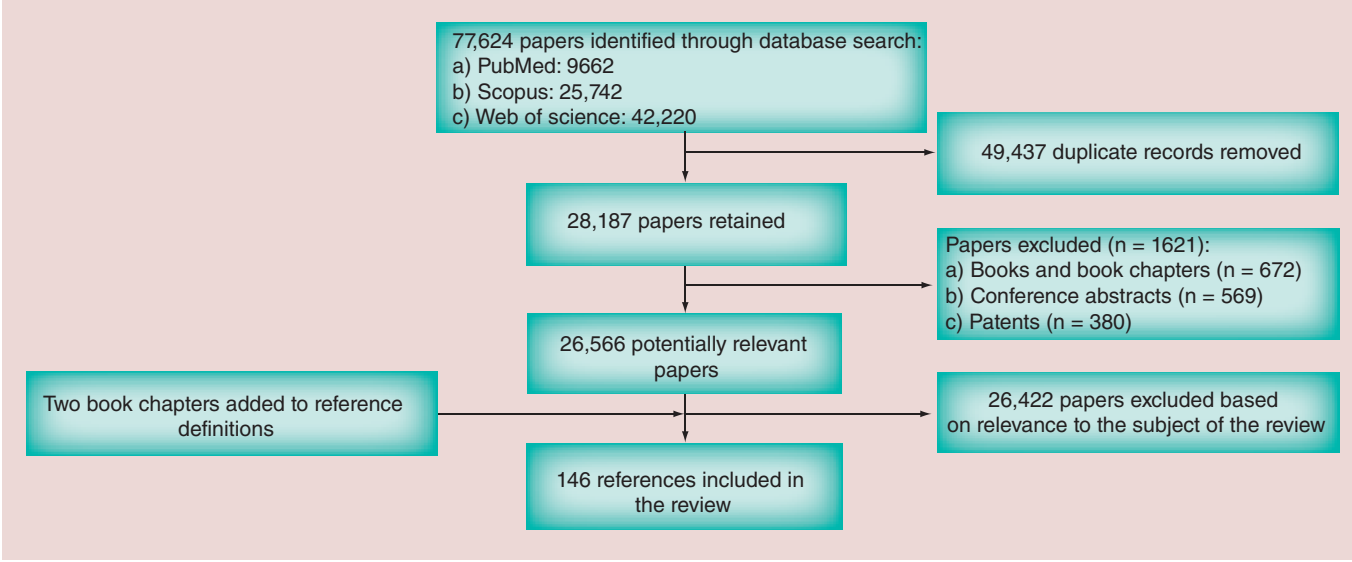

Figure 1. Literature search flowchart. This study adapted the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines. PubMed, Scopus and Web of Science databases were searched, by three independent reviewers, for appropriate studies published up to 1 December 2014, without a lower date limit. Searched keywords included: 'nano', 'nanodiagnostics', 'nanopharmacology', 'nanotoxicology', 'platelets', 'vessel wall interactions', 'endothelium', 'endothelial cells', 'adhesion', 'adhesion molecules', 'aggregation', 'platelet receptors', 'microfluidics' and 'shear stress' in different combinations as per Boolean logic rules. Review of full-text articles was limited to the ones published in English. References of the identified articles were searched manually. Only full text original and review articles (excluding books, book chapters and conference abstracts) strictly pertaining to the subject of this paper, were included in this manuscript. Inclusion or exclusion of studies was performed hierarchically based on the title of the report first, followed by the abstract, and finally by the full text. Finally, from the body of gathered literature, based on our clinical and research experience, the most relevant articles were chosen to form the knowledge base for this review. In the final version of the manuscript, two book chapters were referenced for definitions.
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of vascular injury. In the case of blood (i.e., colloidal suspension containing blood elements) the shearing effect is frequently represented by shear rate – the ratio of shear stress to blood viscosity [10]. Vessel wall shear rates usually range from 300–800 s^{-1} in large arteries, from 500–1600 s^{-1} in arterioles to 10,000 s^{-1} in stenotic vessels [11,12]. With shear rates in the range of 500–5000 s^{-1} platelet adhesion is mainly controlled by the interaction of GPIb-IX-V complex and vWF [10]. This changes when shear rates exceed 5000 s^{-1} as the resulting shear gradients promote ECs to increase vWF secretion and cause GPIb-IX-V-dependent thrombus formation [13]. However, platelet adhesion mediated only by the GPIb-IX-V–vWF interaction is unstable [14], and platelet integrins are necessary for stable adhesion to vWF or fibrinogen [15].

The vWF can be produced either by ECs or released from platelet α-granules. Kanaji *et al.* [16] have shown that in mice either of the two vWF sources is sufficient to support complete hemostasis. Vascular injury, through exposure of extracellular matrix substrates (such as collagen), causes rapid platelet adhesion, followed by activation, secretion and aggregation. These complex interactions are mediated by over 30 different GPs (Table 1) [1].

Vascular endothelium & the platelet–vessel wall interactions

The endothelium consists of a nonthrombogenic monolayer of ECs which lines the circulatory system and together with the basement membrane forms the intima. In an adult human the combined endothelial surface area is approximately 1000 m^2 [17]. The endothelium is not only a physical barrier separating blood components from subendothelial structures, but is also responsible for regulation of coagulation and platelet aggregation, modulation of vascular tone and permeability, as well as fibrinolysis [18].

The luminal surface of the endothelium is covered with a negatively charged glycocalyx, which prevents resting platelets from binding to EC surfaces due to electrical repulsion by negatively charged heparan sulfates [19]. Active platelets overcome this mechanism by releasing heparanase, which degrades the heparan sulfates.

Prostacyclin (PGI_2) is a potent inhibitor of platelet aggregation [20] released by ECs, as demonstrated both in human [21] and animal studies [22]. It is synthesized from arachidonic acid endoperoxides by prostacyclin synthase, and binds to the prostanoid receptor (G protein coupled) on the platelet membrane. Prostacyclin

stimulates the activity of adenylyl cyclase, thus increasing platelet cAMP levels, leading to calcium reuptake and inhibition of platelet aggregation [23].

Nitric oxide (NO) is a labile mediator released by platelets [24] and ECs [25] during platelet–endothelium interactions. It is synthesized from a semiessential amino acid, L-arginine [26] and endowed with vasodilator and antithrombotic properties [27]. It diffuses through the platelet membrane, and binds to the soluble guanylyl cyclase, thus increasing cGMP levels and downregulating platelet adhesion, aggregation and recruitment [27,28]. Both prostacyclin and NO interact with each other to regulate platelet function and vascular tone [29]. Another mechanism by which ECs decrease platelet activation is the surface bound CD39 [30]. The MMP/TIMP/ADAM/ADAMTS system also plays a crucial role in regulation of platelet function [31]. Examples of vascular endothelial pro- and antithrombotic mediators are presented in Table 2 [3].

Not only disruption of endothelial integrity (by exposing collagen, vWF, fibronectin and tissue factor), but also stimulation by increased shear stress, oxidants, endotoxin, TNF and IL-1 can lead to a procoagulative state [32]. Tissue factor released by ECs initiates the coagulation pathway, impairs fibrinolysis by secretion of plasminogen activator inhibitor and reduces the surface expression of thrombomodulin. Furthermore, stimulated ECs attract leukocytes by synthesizing chemokines, and expressing intracellular adhesion molecules linking platelet activation to inflammation [33]. Figure 4A & B shows an overview of molecular regulation of platelet–platelet and platelet–leukocyte–vessel wall interactions.

Diagnostics of platelet activation: role of micro- & nano-fluidic devices for studying platelet–vessel wall interactions

The studies of platelet–vessel wall interactions are of importance for the development of novel antiplatelet drugs. A number of methods have been developed for studying these interactions such as flow chambers (coated with collagen, vWF or lined with ECs) allowing to use varying shear stress rates, microfluidic models of focal vascular injury, miniature parallel-plate perfusion chambers, microfluidic chip-based assays and microdevices to monitor dynamic platelet aggregation in response to strain rate microgradients under flow conditions, among others [3]. Standard methods

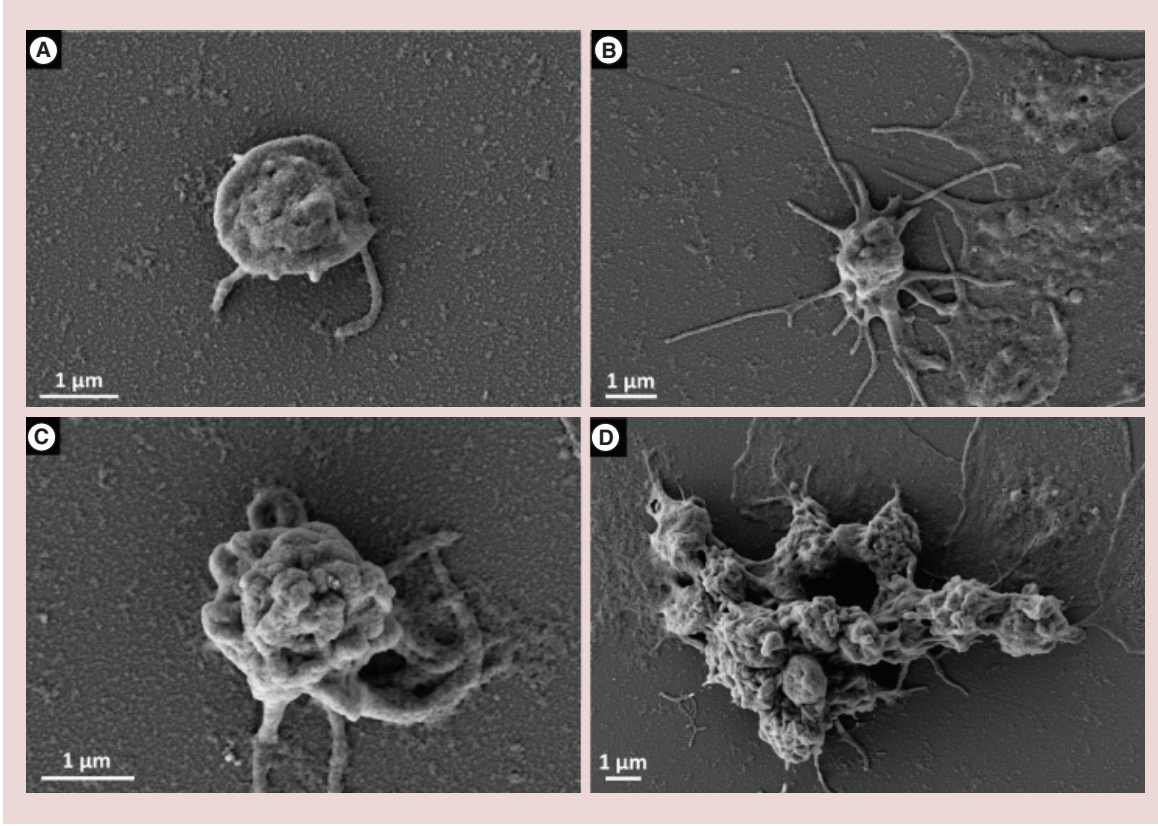

Figure 2. The ultrastructural features of human platelet activation on polyvinyl chloride surface as shown by scanning electron microscopy. Platelet shape changes after activation: **(A)** discoid platelet with short pseudopodia; **(B)** platelet with long pseudopodia interacting with platelets adhering to the surface and showing a 'fried egg' structure; **(C)** small platelet aggregate; and **(D)** platelet aggregate.

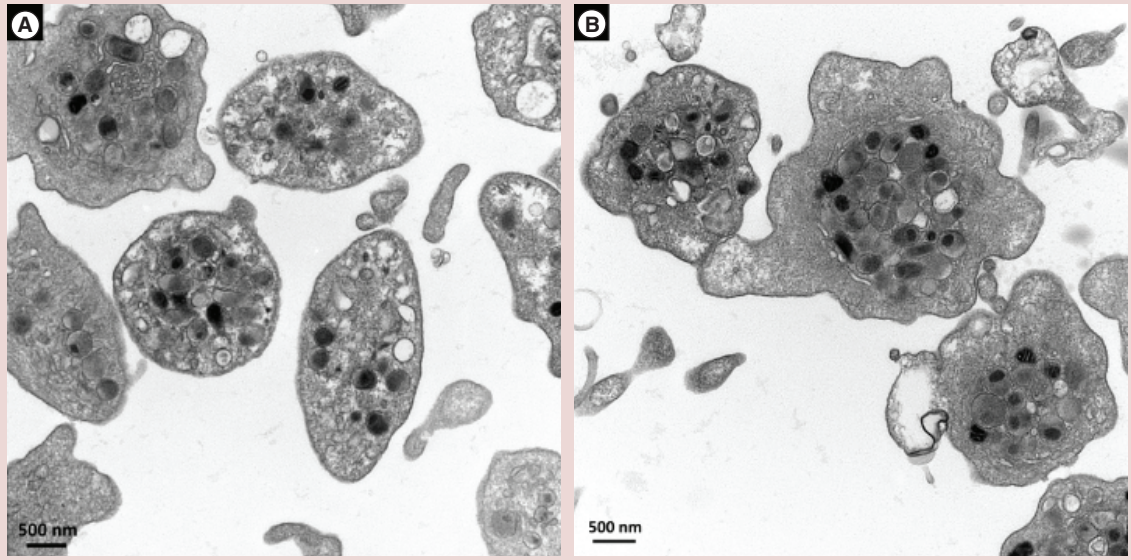

Figure 3. Transmission electron microscopy images of resting and carbon nanotube-activated human platelets. **(A)** Resting platelets showing granules randomly distributed in the cytoplasm and **(B)** centralization of platelet granules (internal contraction) following stimulation with open single wall carbon nanotubes (10 mg/l).

such as light aggregometry, electronic aggregometry, rapid platelet function analyzer or the 'cone and plate' method are not suitable for microvasculature studies [3]. Obtaining microsize blood vessels for *ex vivo* studying of platelet–vessel wall interactions is very challenging due to technical difficulties such as preservation of physiological architecture and function of ECs during and after dissection. However, some authors [34] propose to overcome these difficulties by using *ex vivo* flow chambers precoated with human vWF, and connected to a microfluidic device. Such systems allow simulating and observing shear-dependent thrombus formation in small vessels.

A promising *in vitro* alternative for studying platelet–vessel wall interactions are microfluidic devices. Several platforms have been developed thus far both conventional [35–37] and remodelable [38–41]. Conventional microfluidic devices allow for isolated platelets, platelet rich plasma or whole blood to be perfused for the evaluation of platelet adhesion and aggregation on the endothelialized surfaces under different flow conditions [37] and stimuli [35]. These studies can be valuable to screen for drugs to promote or inhibit platelet adhesion to the endothelium [36]. They can also be used to study the biophysical mechanisms of microvascular occlusion in diseases such as sickle cell anemia and hemolytic-uremic syndrome [37]. However, they are not without their limitations mainly due to the shape of the chamber, which does not resemble, in its cross-section, a typical blood vessel. Though this problem has been solved by introducing cylindrical chambers [42], there still remains the issue of wall rigidity, which prevents the EC layer from distending and remodeling in response to shear stress.

A promising alternative are remodelable microfluidic devices which consist of biomaterials able to respond to shear stress and conventional microfluidics alike. This allows for creating endothelialized structures that support the diffusion of water and gases [32]. Studies by Wong *et al.* [38] and Price *et al.* [39] describe the development of a remodelable microfluidic devices with variable lumen of the tube, and demonstrate that the barrier function of the endothelium can be altered by flow at either very low or very high shear stresses [39], or via elevating cytoplasmic cAMP [38]. In their recent studies Morgan *et al.* [40] and Zheng *et al.* [41] have developed a microchannel network and seeded it with ECs. The channels were formed within a matrix of native type I collagen providing appropriate stiffness and allowing for remodeling by ECs through matrix degradation or deposition. This system gives the ability to control cellular, chemical, and physical stimuli, and allows for the ECs to grow and mature obtaining full barrier functions within a week from seeding.

Another interesting concept for the study of platelet adhesion and aggregation under flow is the use of quartz crystal microbalance with dissipation (QCM-D) [3]. It works through applying an alternating electric current to the quartz crystal sensor and measuring its resonance frequency and energy dissipation. Our group [43] has shown that the QCM-D is able to measure nanoparticle (NP)-induced plate-

Table 1. Platelet membrane receptors and their pharmacological inhibitors.

Receptor name	Receptor type	Receptor agonists	Receptor role	Inhibitors
P-selectin	Selectin	PSGL-1	Adhesion	Heparin, sulfated trimannose C-C-linked dimers
GPIb	Integrin	vWF	Adhesion	Anti-vWF mAb, anti-GPIb mAb; Bivalent nanobody ALX-0081 for the vWF A1 domain
GPIV	Nonintegrin	Collagen type I	Adhesion	
GPIa/IIa	Integrin	Collagen types I–VIII	Adhesion	
GPIb-V-IX	Integrin	Collagen types I, III and VI	Adhesion	
GPIc/IIa	Integrin	Collagen	Adhesion	
GPVI	Nonintegrin	Collagen types I–III	Activation	JAQ1 (mAb tested in mice), anti-GPVI, GPVI-Fc fusion protein
$P2Y_1$	G protein coupled	ADP	Aggregation	
$P2Y_{12}$	G protein coupled	ADP	Aggregation	Irreversible (ticlopidine, clopidogrel, prasugrel) Reversible (ticagrelor, cangrelor, elinogrel)
TPα and TPβ	G protein coupled	TXA_2	Aggregation	Aspirin, terutroban (selective)
PAR-1 and PAR-4	G protein coupled	Thrombin	Aggregation	Atopaxar
GPIIb/IIIa	Integrin	Fibrinogen	Aggregation	Abciximab, eptifibatide, tirofiban

mAb: Monoclonal antibody; PAR: Protease activated receptor; TP: Thromboxane receptor.

let aggregation at concentrations that are undetectable by light aggregometry and flow cytometry. Using this technique, NP-induced platelet aggregation can be quantified in real time under flow conditions and later directly analyzed on the sensor using microscopic techniques (Figure 5).

Interestingly, platelets can also interact with cancer cells and these interactions, known as tumor cell-induced platelet aggregation (TCIPA), play an important role in carcinogenesis and blood-borne cancer spread [44]. Our group has described a microfluidic method with an ultrasound standing wave trap to study TCIPA [45].

Nanodiagnostics of platelet–vessel wall interactions

Nanodiagnostics is defined as the use of nanotechnology for clinical diagnostic purposes [46]. Nanomaterials allow for more sensitive and specific diagnosing of pathologies, and are currently being used as contrast agents for molecular and whole cell imaging *in vitro* and *in vivo*.

Contrast-enhancing NPs are essential in the field of molecular imaging. While conventional imaging modalities such as computer tomography and MRI allow for the visualization of anatomical and pathological consequences of diseases. Molecular imaging is based on the molecular and cellular fingerprints of the disease, requiring disease-specific molecular targets as well as target-specific NPs. Furthermore, these NPs should allow *in vivo* visualization with sufficient accuracy. Though MRI is constantly evolving, its sensitivity is still low without the proper contrasting agents [47]. The most common approaches to enhance the sensitivity of MRI include biomimetics, peptides or monoclonal antibodies with gadolinium as the paramagnetic contrast agent [47]. A newer approach, building on the discoveries of nanoscience, uses iron oxide NPs as contrasting agents [47,48]. These include: very small iron oxide particles (VSOPs; diameter <10 nm), ultrasmall superparamagnetic iron oxide particles (USPIOs; diameter 10–40 nm), and small superparamagnetic iron oxide particles (SPIOs; diameter 50–150 nm) [49]. Small and ultrasmall PIOs are currently marketed [47] and guarantee a strong T2 contrast at nanomolar concentrations with degradation occurring through physiological iron-based pathways within 7 days [50]. Untargeted SPIO and USPIO NPs have long been clinically used to target cells associated with both the reticular endothelial and monophagocytic systems [47].

Ruehm *et al.* [51] describe the use of USPIOs as a marker of atherosclerosis-associated inflammatory changes in the vessel wall before luminal narrowing is present. The authors, using a rabbit model have shown through the use of MR angiography, that USPIOs can demonstrate changes in the thoracic aorta of hyperlip-

Table 2. Vascular endothelial pro- and anti-thrombotic mediators.

Vascular endothelial mediators	Action
Prothrombotic	
Tissue factor	Coagulation
vWF	Platelet adhesion and aggregation
Plasminogen activator inhibitor	Fibrynolysis inhibition
Antithrombotic	
Prostacyclin, nitric oxide, CD39	Platelet activation inhibition
Thrombomodulin, heparin/dermatan sulfate, tissue factor pathway inhibitor	Coagulation inhibition
Tissue-plasminogen activator	Modulation of fibrynolysis

idemic animals, whereas traditional contrasting agents fail to detect any abnormalities. This was confirmed by *ex vivo*, histopathological, and electron microscopy assessments. The authors concluded that USPIOs are phagocytosed by macrophages in atherosclerotic plaques of the aortic wall of hyperlipidemic rabbits in a quantity sufficient to be detectable by MRI.

P-selectin is an adhesion molecule, expressed at the surface of ECs and platelets upon activation, which mediates leukocyte rolling and leukocyte trapping within the thrombus [33]. In a recent study Suzuki *et al.* [52] created fucoidan coated USPIOs (USPIO-FUCO) that *in vitro* bind to immobilized P-selectin. Fucoidan is a natural (derived from algae) sulfated polyfucose with high affinity for activated platelets. Surface plasmon resonance confirmed that both fucoidan and USPIO-FUCO have a high affinity to P-selectin allowing for the imaging of intraluminal thrombi. Furthermore, when tested in a rat aneurysm model, using MRI, and histological analysis, it was confirmed that USPIO-FUCO can detect thrombi in rat aneurysms with high sensitivity showing that these NPs might be an efficient molecular imaging agent for MRI. Importantly, fucoidan safety has been investigated in recent years in cosmetics and food supplements, without any adverse effects reported in animals or humans [53]. Jacobin-Valat *et al.* [47] also imaged P-selectin in atherosclerosis-prone arteries using dextran/iron oxide NPs conjugated with PEG (PEGylated) (versatile ultrasmall superparamagnetic iron oxide [VUSPIO]) labeled with rhodamine and coupled to the antihuman VH10 P-selectin antibody. Both flow cytometry and histology results highly correlated with MRI, showing that VH10–VUSPIO NPs are a promising imaging modality able to identify the early stages of atherosclerosis.

In addition to P-selectin other adhesion molecules involved in the development of atherosclerotic lesions can be targeted by NP imaging. Michalska *et al.* [54] used USPIOs functionalized with VCAM-1 binding peptide to visualize early and advanced atherosclerotic lesions of apolipoprotein E-deficient mice. They demonstrated that VCAM-1 binding protein functionalized USPIOs are capable to target atherosclerotic plaques, allowing visualization using MRI and surface-enhanced coherent anti-stokes Raman scattering microscopy.

Phosphatidylserine (PS) is yet another molecular target which may be used for NP imaging. This lipid is expressed on the surface of apoptotic cells, as well as platelets during advanced stages of activation and is known to bind to AnxA5. Prinzen *et al.* [55] demonstrated the use of AnxA5-functionalized quantum dots (QDs) in MRI or fluorescent methods for imaging apoptosis and platelet activation. This study tested AnxA5-functionalized QD NPs with eight complexes of gadolinium-DTPA attached that were successfully analyzed using MRI.

In addition to magnetic NPs, dendrimers have also been used for molecular imaging of platelet activation. Woolley *et al.* [56] conjugated stable, bright, dye-doped silica NP surface functionalized with PAMAM dendrimers with platelet activation-specific antibodies. Using a single-step protocol the authors demonstrated highly specific platelet labeling with the distribution of antibody-conjugated NPs matching that expected for the platelet GPIIb/IIIa receptor.

Finally, Davies *et al.* [57] developed a pH-controlled live platelet labeling system using luminescent tracer coated gold NPs combined with a pH low insertion peptide into platelets. The results showed that these NPs enter platelets at pH 6.5 but not pH 7.4. This entry is mediated by peptide translocation across the membrane. The complex gives a clear signal from the luminescent probe and may potentially be used to track live platelets.

Platelets themselves may also be labeled and imaged by NPs. Oldenburg *et al.* [58] used optical coherence tomography (OCT) to detect rehydrated, lyophilized platelets labeled with US FDA-approved SPIOs. These

platelets bind to sites of vascular injury, demonstrating their potential clinical usefulness. Aurich *et al.* [48] also developed magnetic labeling of human platelets *in vitro*. Using SUPIOs with a carboxydextran shell (both FDA- and EMA-approved), without any additional ligands, the authors were able to demonstrate labelling of 98% of platelets, with SUPIOs predominantly located inside the platelet granules as confirmed by fluorescence microscopy and transmission electron microscopy. These SUPIO-labeled platelets could be assayed by MRI. Finally, von zur Muhlen *et al.* [59] used larger MPIOs (1 μm in size) with a single chain antibody ligand to selectively bind to ligand-induced binding sites on activated GP IIb/IIIa (the most abundant platelet receptor) to image human and mouse carotid thrombi. The MPIOs allowed *in vivo* MRI of activated platelets with excellent contrast properties capable of monitoring the effects of thrombolytic therapy.

Currently, nanodiagnostics allow only for *in vivo* clinical monitoring of gross pathologies like atherosclerotic plaques [60]. The main limitation preventing *in vivo* monitoring of individual platelet–vessel wall interactions is the spatial resolution and/or the restricted penetration depths of the imaging modalities employed [61]. High quality noninvasive methods like MRI have a spatial resolution of 10–100 μm with an unlimited penetration depth, while OCT (an invasive imaging method) has a spatial resolution of approximately 10 μm but a penetration depth of only 2–3 mm [61]. With an individual platelet size of about 2 μm, this still confines monitoring of individual platelet–vessel wall interactions [55–57] to *ex vivo* and *in vitro* laboratory methods. However, while currently used noninvasive imaging modalities are limited in their spatial resolution or penetration depth by physical principles [61], invasive monitoring is quite close to achieving the required parameters to visualize *in vivo* single platelet-EC interactions.

Desirable effects of nanomaterials in platelet–vessel wall interactions

In therapeutics, pharmaceuticals exert a broad spectrum of effects spanning from desirable (therapeutic) to undesirable including nondeleterious (side effects) and deleterious (toxic effects). It is important to realize that a pharmaceutical agent typically exerts numerous effects, but usually one or very few are sought as the primary objective of treatment [62]. The following sections will review both desirable and undesirable effects of nanomaterials. We have recently proposed the term of nanopharmacology as a branch of pharmacology that applies the findings of nanotechnology to drug discovery and development in order to maximize the therapeutic efficacy and reduce toxicity of new drug and treatment entities [63]. This can be achieved by targeted delivery of drugs to specific sites in a controlled manner [64], development of nanodrugs [65] or therapeutic nanostructures. Further information on NP interactions with blood elements besides platelets can be found in the review by Ilinskaya and Dobrovolskaia [66].

Platelet-mimicking NPs that stimulate platelet–vessel wall interactions & arrest bleeding

Platelet hemostasis plays a crucial role in maintaining vascular integrity, which may be compromised as a result of accidental injury or disease. Hemostatic reactions start from the formation of occlusive platelet plug and lead to restoration of vessel integrity and physiological blood flow. Recently, novel nanostructures called platelet-mimicking NPs have been designed to mimic some of platelet hemostatic functions, in particular platelet adhesion via GPIb and aggregation via GPIIb/IIIa.

Rybak and Renzulli [67] introduced for the first time platelet-mimicking NPs. They combined a platelet membrane deoxycholate extract (containing, among other proteins, GPIb, GPIIb/IIIa and GPIV/III) with sphingomyelin creating a 'plateletsome'. These small liposomes showed excellent hemostatic properties halting bleeding in a thrombocytopenic rat tail wound model. Direct wound infusion was more efficient than systemic administration of plateletsomes. Postmortem analysis of treated animals revealed no pathologic thrombi in any of the organs. Plateletsomes, when tested *in vivo* in rabbits did not cause intravascular coagulation. Also no increased platelet aggregation *in vitro* was observed.

A similar approach was employed by Modery-Pawlowski *et al.* [68]. The authors developed surface-enhanced liposomes with collagen- and vWF-binding peptides to mimic platelet adhesion, and a fibrinogen-mimetic peptide to promote platelet aggregation. By modulating the densities of the peptides under varying shear flow the authors were able to optimize the adhesion and aggregation properties of platelet-mimicking liposomes. These constructs showed significantly better hemostatic properties compared with 'adhesion-only' or 'aggregation-only' models.

Nishikawa *et al.* [69] also building on the 'platelet-mimicking liposome' experience devised fibrinogen γ-chain-coated, ADP-encapsulated liposomes (H12-ADP-liposome) that accumulate at bleeding sites via GPIIb/IIIa interaction with activated platelets and augment platelet aggregation by releasing ADP. The administration of these liposomes to thrombocytopenic rabbits with noncompressible liver hemorrhage rescued all animals. On postmortem evaluation

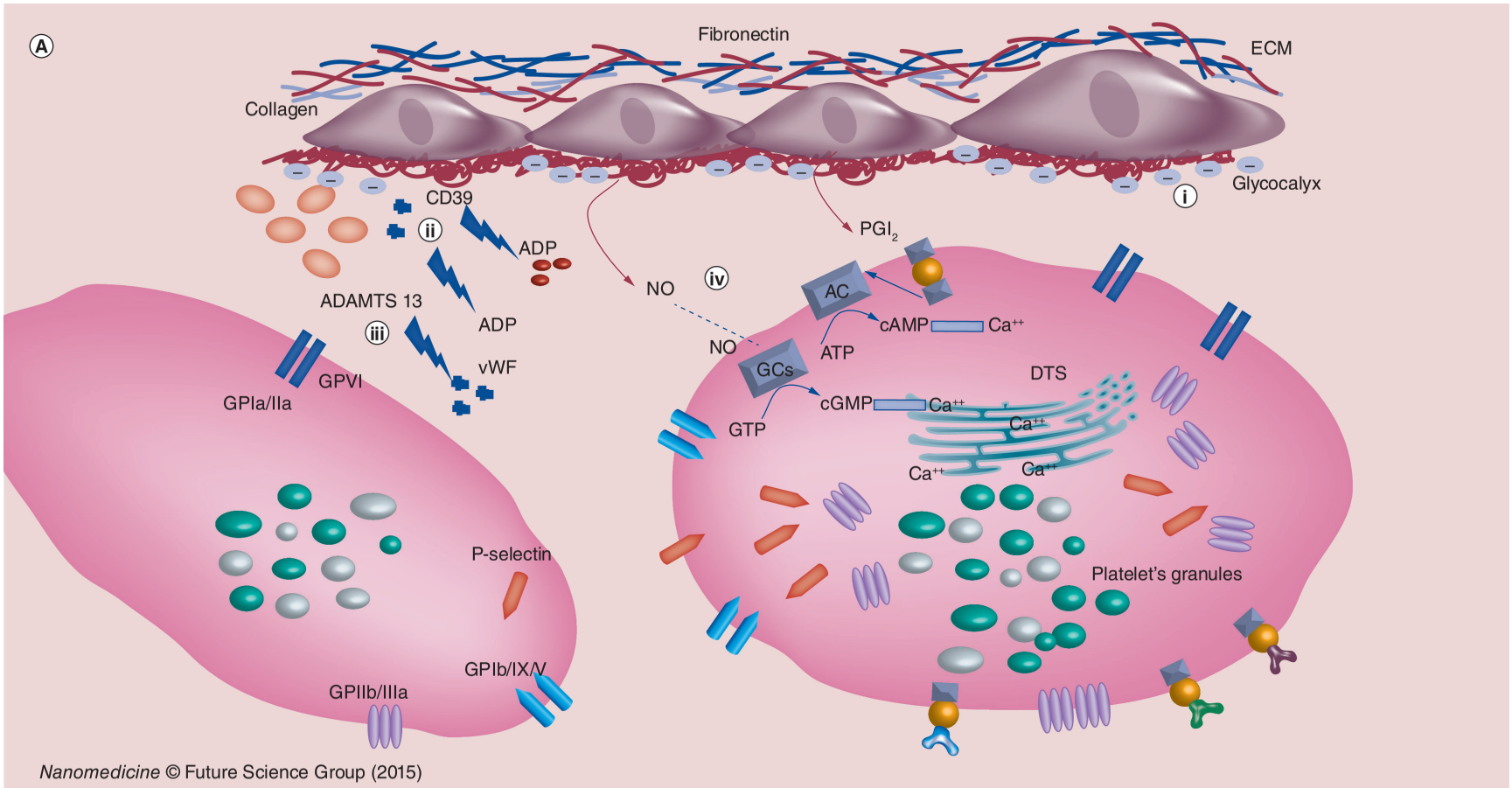

Figure 4. Platelet–platelet and platelet–leukocyte–vascular wall interactions (for [A], see above; for [B], see facing page). (A) The resting platelet and the antithrombotic properties of the vascular endothelium. In the absence of any activating stimulus, platelets circulate in a resting state. Both, a combination of different factors and an intact and healthy endothelium play an essential role in preventing the attachment of platelets and proteins involved in the coagulation cascade, and therefore, platelet aggregation. The negative charge on the surface of the endothelial cells that repels negatively charged platelets **(i)**. The presence of CD39, an ADPase, on the surface of endothelial cells that cleaves any small amounts of ADP that otherwise could activate the surrounded platelets **(ii)**. ADAMTS 13, that is implicated in the proteolytic cleavage of the vWF **(iii)**. NO and PGI_2 that are generated and released by endothelial cells. The actions of PGI_2 are mediated via a G-protein coupled receptor that activates adenylyl cyclase converting ATP into cAMP. Nitric oxide diffuses freely across membranes and stimulates the activity of guanylyl cyclase resulting in an increase of intracellular cGMP levels **(iv)**.

AC: Adenylyl cyclase; ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs; DTS: Dense tubular system; ECM: Extracellular matrix; GCs: Soluble guanylyl cyclase; GP: Glycoprotein; NO: Nitric oxide; PGI_2: Prostacyclin; TF: Tissue factor.

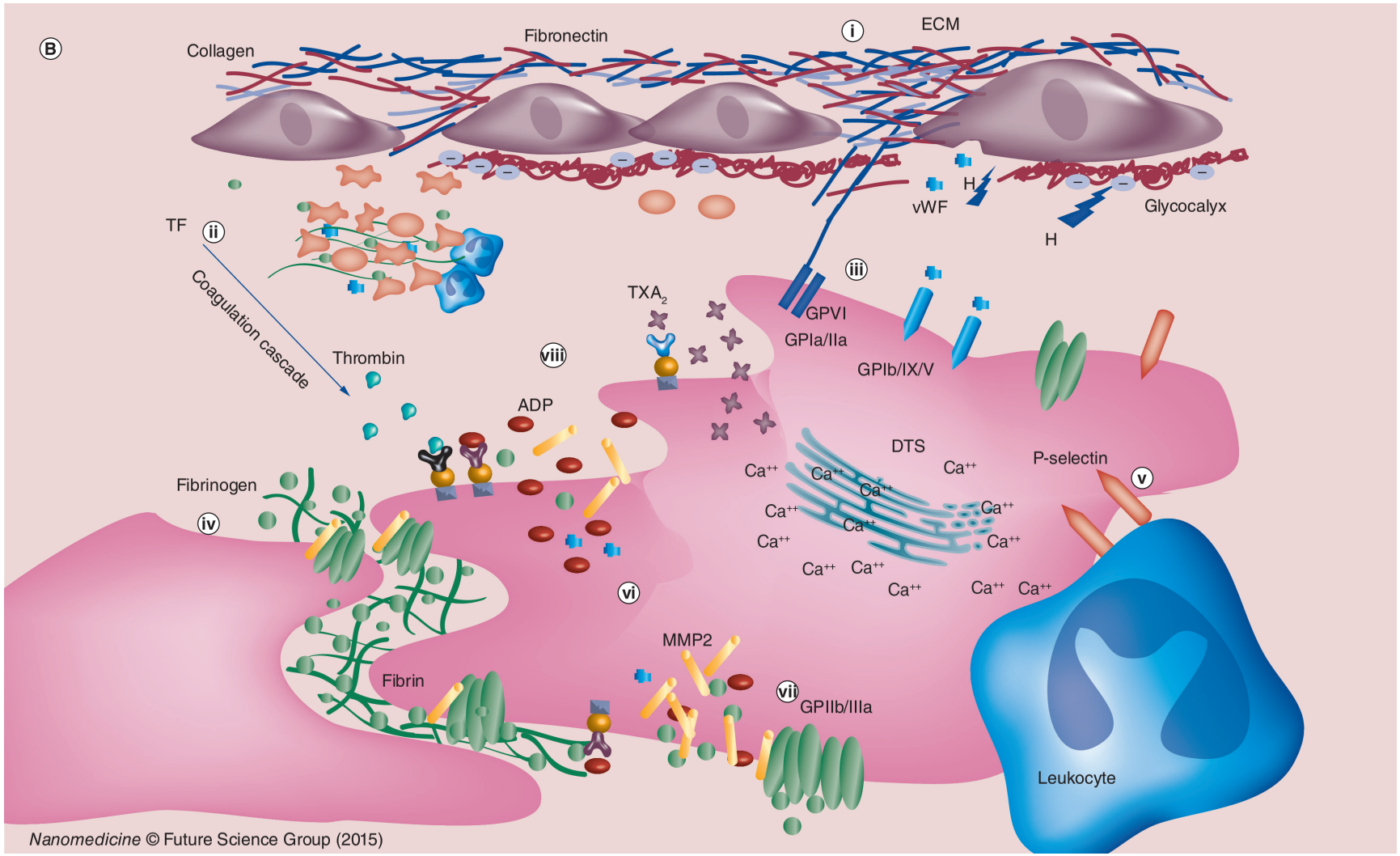

Figure 4. Platelet–platelet and platelet–leukocyte–vascular wall interactions (for [B], see above; for [A], see facing page). **(B)** The activated platelet: platelet–platelet and platelet–leukocyte–vascular wall interactions. The main trigger for the formation of a hemostatic plug after vascular injury is the loss of the endothelial cell barrier between extracellular matrix components and flowing blood **(i)**. Collagen when exposed to the vascular lumen and vWF are responsible for the accumulation and activation of platelets. The exposed TF is responsible for the generation of thrombin stimulating the coagulation cascade **(ii)**. Thrombin not only converts fibrinogen to fibrin but also activates platelets. Collagen and vWF bind to platelet GP receptors **(iii)** and platelets spread on the extracellular matrix, forming a monolayer. In order for platelets to accumulate on the initial platelet monolayer the activation of GPIIb/IIIa receptors is required to promote the interactions with adjacent platelets and fibrinogen **(iv)**. P-selectin, which is mainly stored in alpha-granules, also translocates to the membrane surface mediating platelet–leukocyte interaction **(v)**. The recruitment of additional platelets is mediated by the release or secretion of TXA_2, ADP, and MMP-2. Thrombin is produced on the surface of activated platelets. The release of vWF, fibrinogen, and fibronectin from alpha-granules also facilitates blood coagulation **(vi)**. MMP-2 is translocated to the platelet membrane where it co-localizes with the GPIIb/IIIa receptor **(vii)**. Thrombin, TXA_2 and ADP act via G-protein-coupled receptors **(viii)**. The activation of G-protein-coupled receptors results in the activation of signalling pathways and mobilization of cytosolic Ca^{++} that induces the reorganization of the cytoskeleton, platelet shape change and secretion of platelet granules.

AC: Adenylyl cyclase; ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs; DTS: Dense tubular system; ECM: Extracellular matrix; GCs: Soluble guanylyl cyclase; GP: Glycoprotein; NO: Nitric oxide; PGI_2: Prostacyclin; TF: Tissue factor.

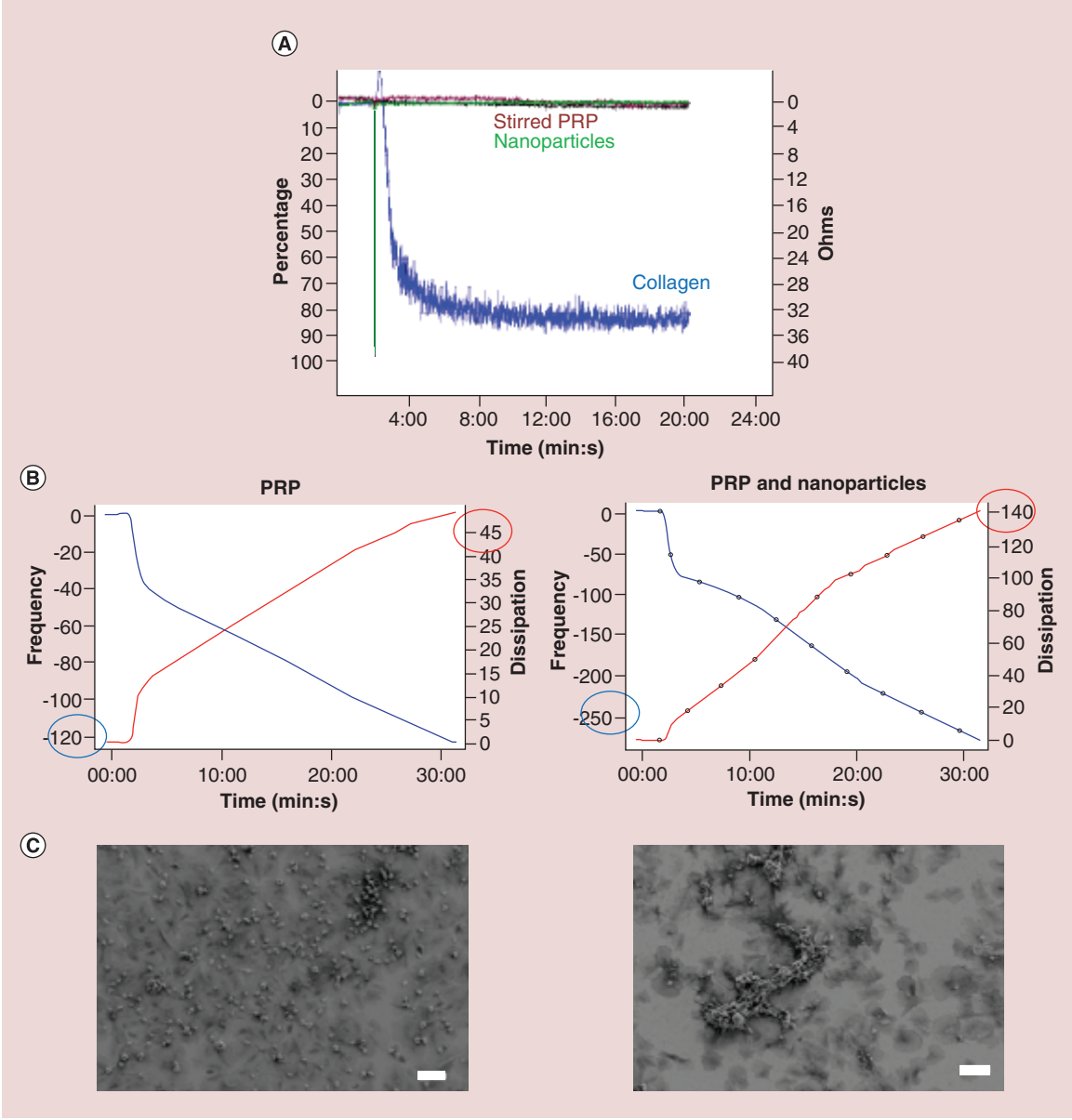

Figure 5. Effect of metal nanoparticles in platelet aggregation as measured by light aggregometry and quartz-crystal-microbalance with dissipation. **(A)** Nanoparticle-induced platelet aggregation is not detected using a light aggregometer: representative traces from aggregometry studies show no aggregation when platelet-rich-plasma is incubated in the presence of 5 μg/ml of metal nanoparticles (green line). Collagen-aggregated platelets (blue line) are used as a positive control; stirred platelets in the absence of nanoparticles (purple line) are used as negative control. **(B)** Nanoparticle-induced platelet aggregation is detected using QCM-D: representative traces from the third overtone recorded by the device show the effects of the perfusion of metal nanoparticles in platelet-rich-plasma. The perfusion of metal nanoparticles at 5 μg/ml in platelet-rich-plasma induce a larger decrease on frequency (blue line, left axis) and increase on dissipation (red line, right axis) when compared with platelet-rich-plasma in the absence of nanoparticles. **(C)** Representative micrographs of the surface of the sensors as viewed by scanning electron microscopy corroborate an increased accumulation of platelet aggregates following perfusion of platelet-rich plasma in the presence of metal nanoparticles.
PRP: Platelet-rich plasma.

H12-ADP-liposome administration did not cause macro- or microthrombi in the lungs, kidneys or liver of the treated animals. Taguchi *et al.* [70] investigated the pharmacokinetic properties of H12-ADP-liposomes showing that the predicted half-life of these constructs in humans will be approximately 96 h, with acceptable biodegradable properties for a synthetic platelet substitute.

Aneslmo *et al.* [71] presented yet another interesting way to produce platelet-mimicking NPs. Spherical

polystyrene NPs, used as templates, were coated with complementary layers of polyallylamine hydrochloride and bovine serum albumin. Next, the core was removed, leaving flexible discoidal NPs, which were covered with collagen-binding peptide, vWF binding peptide, and linear fibrinogen-mimetic peptide. Thus, the produced NPs mimicked blood platelets in their four key features – discoidal morphology, mechanical flexibility, biophysically and biochemically mediated aggregation and heteromultivalent presentation of ligands that mediate adhesion. Under physiological flow conditions these constructs exhibited site-selective adhesive and platelet-aggregatory properties. *In vivo* studies in a mouse model have shown that these platelet-mimicking NPs accumulate at sites of vascular injury and reduce bleeding time by 65%.

Okamura *et al.* [72] pursued another concept of preparing 'platelet-mimicking' constructs, by creating poly(lactic-co-glycolic acid) (PLGA) free standing nanosheets conjugated with the H12 peptide. These disc-shaped nanosheets had a large contact area for the targeting site, rather than a conventional small contact area seen in spherical carriers. Under flow conditions these NPs interacted with activated platelets adhered on a collagen surface, and showed platelet thrombus formation in a 2D spreading manner.

Doshi *et al.* [73] engineered polymeric polystyrene micron-sized particles which resembled natural platelets in size, discoid shape and flexibility. To increase resemblance the authors used actin (which forms the natural platelet cytoskeleton) as the structural protein. The particles were coated with vWF-A1 ligand to target the GPIbα on platelets. The particles were recruited to platelet aggregates in an *ex vivo* model of platelet aggregation showing their diagnostic, hemostatic and pharmacological potential.

Finally, Bertram *et al.* [74] devised nanospheres with PLGA-poly-L-lysine (PLGA-PLL) cores to which they PEGylated arms terminated with RGD (Arg-Gly-Asp) functionalities. The RGD motif recognizes the activated, but not resting, conformation of GPIIb/IIIa; therefore, *in vitro* nanospheres did not bind to resting platelets, and did not activate them without the addition of ADP. *In vivo*, in a rat femoral artery injury model, platelet-mimicking nanospheres interacted preferentially with activated platelets due to RGD functionalization, and actively arrested bleeding.

It is worth to emphasize that biodegradable platelet-mimicking NPs such as PLGA that have half-life *in vivo* resembling that of circulating platelets (a few days) may be more biocompatible than non-biodegradable that are likely to persist longer in the body [75].

Nanodrugs & nanodelivery systems in the treatment of vascular thrombosis

Vascular thrombosis complicates the course of arterial or venous pathologies such as atherosclerosis of coronary and cerebral arteries or varicose veins. Anticoagulant, thrombolytic and antiplatelet drugs are clinically used for the prevention and management of these complications [76]. The main side effects encountered with this treatment are due to bleeding. Selective targeting of thrombotic areas, thus sparing the healthy part of the vasculature, can potentially be achieved using platelet activation-selective drug delivery systems or nanodrugs.

Nanocarrier-platelet biocompatibility is an important characteristic when designing drug-releasing pharmaceuticals including antiplatelet drugs. Li *et al.* [77] first investigated platelet biocompatibility of PLGA, chitosan and PLGA-chitosan NPs, showing that *per se* all the tested NPs at concentrations <10 μg/ml did not modify platelet aggregation, demonstrating that they may be used for the delivery of active molecules to the bloodstream.

Selective delivery of antiplatelet drugs to activated, but not resting platelets can be accomplished by targeting platelet activation-specific receptors including the activated GPIIb/IIIa and P-selectin. Srinivasan *et al.* [78] developed liposomes modified with cyclic RGD peptide ligands to specifically target the activated GPIIb/IIIa. Microscopy results showed successful platelet-targeting by these peptide-modified liposomes. Moreover, Zhu *et al.* [79], using P-selectin glycoprotein ligand 1-mimicking liposomes with a PEG spacer, have shown that adding the PEG spacer increases the liposome–P-selectin binding capacity 22-fold. The modified liposomes bound to platelets, making them potential nanocarriers for drugs targeting platelets at sites of vascular injury.

Xu *et al.* [80] developed a multiligand poly(L-lactic-co-glycolic acid) platelet-mimicking NPs (spherical, ~200 nm) conjugated with GPIb, and transactivating transcriptional peptide. The PLGA-PEG-GPIb conjugate strongly adhered to P-selectin or vWF-coated surfaces, and was internalized by ECs. Treatment of injured rat carotid arteries with these multiligand-NPs suppressed neointimal stenosis more than unconjugated NPs, showing potential clinical use to target inflamed ECs and inhibit inflammation, as well as subsequent stenosis.

Kona *et al.* [81] developed biodegradable PLGA NPs and conjugated them with glycocalicin (external fraction of platelet GPIbα) to specifically target ECs. This process significantly increased NP adhesion to P-selectin- and vWF-coated surfaces as well as NPs uptake by activated ECs under shear stress. The authors demon-

strated the suitability of their system to deliver drugs directly to ECs (at the site of vascular injury) under flow conditions. The study by Kona *et al.* [81] is in keeping with an earlier study of Lin *et al.* [82] who conjugated fluorescent carboxylated polystyrene NPs with glycocalicin, showing significantly increased particle adhesion on P-selectin-coated surfaces and cellular uptake of NPs by activated ECs under physiological flow conditions.

Another NP that could act as a potential drug in antithrombotic therapy is the PAMAM dendrimer G4-Arginine-Tos derivative, which Duran-Lara *et al.* [83] have shown to be hemocompatible and produce desirable antiplatelet and antithrombotic effects.

In addition, NPs can target ECs; however, changes in the local flow microenvironment and shear stress can affect targeting. Han *et al.* [84] examined endothelial internalization of nanocarriers targeted to PECAM-1. ECs do not internalize antibodies to marker PECAM (CD31), yet internalize multivalent NPs coated with PECAM antibodies. The authors showed that EC adaptation to chronic flow, manifested by cellular alignment with flow direction and formation of actin stress fibers, inhibited anti-PECAM/NP endocytosis. By contrast, acute flow without stress fiber formation stimulated anti-PECAM/NP endocytosis.

Shi *et al.* [85] devised a tissue factor targeted nanomedical system for thrombi-specific drug delivery. They conjugated PEG-PLGA NPs with EGFP-EGF1, a tissue factor targeting protein. The obtained construct significantly facilitated uptake via the EGF1/TF mediated endocytosis pathway, and was assessed as safe by *in vitro* and *in vivo* testing.

As expected [86] leukocytes and red blood cells may affect flow-mediated margination of polymeric nanospheres and microspheres to ECs.

Early administration of thrombolytic drugs (tPA and congeners) to dissolve occlusive thrombi is one of the cornerstones of the therapy of thromboembolic diseases including ischemic stroke [87]. Such thrombi increase shear stress by one to two orders of magnitude, from below ~70 dyne/cm^2 in normal vessels to >1000 dyne/cm^2 in highly constricted arteries [11,12]. Korin *et al.* [88] devised a biomimetic strategy employing high shear stress caused by vascular narrowing as a targeting mechanism to deliver thrombolytic drugs to obstructed blood vessels. The authors developed PLGA microparticles (1–5 μm) that were covered with tissue plasminogen. When exposed to high shear stress the particles break up into nanoscale components resulting in plasminogen activation and stimulation of thrombolysis. Importantly, this strategy allows to lower the required thrombolytic drug dose over 100-fold, when compared with currently used protocols, thus limiting the likelihood of hemorrhagic side effects of thrombolysis.

The platelet 'inner targeting mechanism' can be also used as a drug delivery system for clotting factors, thrombolytic or anticancer drugs [89]. Due to their unique ability to target sites of vascular injury, as well as cancer cells, drug-loaded platelets were successfully used to treat hemophilia-related bleeding (platelets loaded with factor VIII) [90] and demonstrated better antitumor results in treating Ehrlich ascites carcinoma bearing mice than the free drug (doxorubicin-loaded platelets) [91].

Further information regarding mechanisms of vascular targeting using nanocarriers can be found in the review by Howard *et al.* [92].

Undesirable & toxic effects of nanomaterials in platelet–vessel wall interactions

The ever growing presence of nanotechnology and related disciplines such as nanomedicine has also increased the exposure of humans to nanosized particles via ingestion, cutaneous uptake, injection and inhalation [75]. In addition, as with all new diagnostics and therapeutics there are safety concerns associated with the use of nanomaterials. This gave rise to nanotoxicology, which deals with safety evaluation of nanomaterials [93,94]. The nano size of nanomaterials allows them to easily pass through cell membranes and other biological barriers, therefore, nanomaterials can be easily internalized by living organisms and cause cellular dysfunction [95]. Additionally, because of their unique properties, including high surface-to-volume ratios, nanomaterials are reactive or catalytic, and thus can be potentially toxic. All of the above warrants studies on the safe use of NPs.

Nanomaterials once in the bloodstream can directly interact with platelets, and ECs changing their properties and platelet–EC interactions [75]. It is important to realize that nanomaterials, in addition to the class-common nanoproperties, are endowed with their own physicochemical and biological characteristics. The following section will review the vascular toxicity profile of the major groups of nanomaterials.

Silica NPs

Both bare and functionalized silica NPs have been tested in different studies. Tavano *et al.* [96] investigated the effects of uncapped and PEGylated silica NPs on platelet–endothelial function and the coagulation cascade. They found that PEGylation reduces the ability of silica NPs to stimulate aggregation yet failed to attenuate coagulation responses. The mechanism of platelet-activator effects of silica NPs could be related to

the ability of these materials to generate bioactive NO by NO synthase. Indeed, Corbalan *et al.* [97,98] investigated the direct influence of amorphous silica NPs on the viability of the vascular endothelium (Figure 6) and endothelial and platelet (NO)/($ONOO^-$) balance and aggregation. The EC exposure to amorphous silica NPs induced platelet aggregation (upregulating P-selectin expression and GPIIb/IIIa activation) with a low ratio of NO to $ONOO^-$, indicative of enhanced O_2^- radical generation. Interestingly, the extent of cell damage decreased with increased size of silica NPs. Amorphous silica NPs have a noncrystalline structure, a high surface-area-to-volume ratio and a large negative surface charge. The high surface area-to-volume ratio correlates inversely with size, and favors the formation of a 'protein corona' which, along with fluid characteristics and particle surface area and zeta potentials, can affect the formation of NP agglomerates in the biological environment, and thus may influence NP toxicity [99,100]. This inverse correlation between size and NP toxicity has also been reported, among other NPs, for iron oxide magnetic NPs [101], and titanium oxide NPs [102].

Carbon NPs

The translocation of combustion-derived particulate matter from lungs to the systemic circulation could underlie cardiovascular toxicity associated with environmental exposure to these particles [103,104].

In 2005, our group made a pioneer discovery showing that carbon NPs have the ability to stimulate platelet aggregation and vascular thrombosis [105]. We investigated the effects of a variety of engineered carbon NPs including nanotubes (MWNT and SWNT), C60 fullerenes (C60CS) and mixed carbon nanoparticles (MCN) on platelet aggregation and compared them with standard urban particulate matter (SRM1648). Carbon particles, except C60CS, stimulated platelet aggregation (MCN ≥ SWNT > MWNT > SRM1648) and accelerated the rate of vascular thrombosis in rat carotid arteries with a similar rank order of efficacy. Interestingly, NP-induced aggregation was inhibited by prostacyclin and NO donor S-nitroso-glutathione, but not by aspirin. The platelet-activator activity of carbon nanotubes was later confirmed by Guidetti *et al.* [106] and Bihari *et al.* [107] which also showed that subthreshold amounts of carbon NPs can synergize with each other to amplify thrombosis. Recently, we have investigated the hemocompatibility of multiple wall nanotubes bound to polyvinyl chloride (one of the most relevant medical surfaces) and found that the presence of nanotubes resulted in platelet activation *in vitro* and *in vivo* in rabbit model of extracorporeal circulation [108], an effect prevented by potent platelet activation inhibitor iloprost.

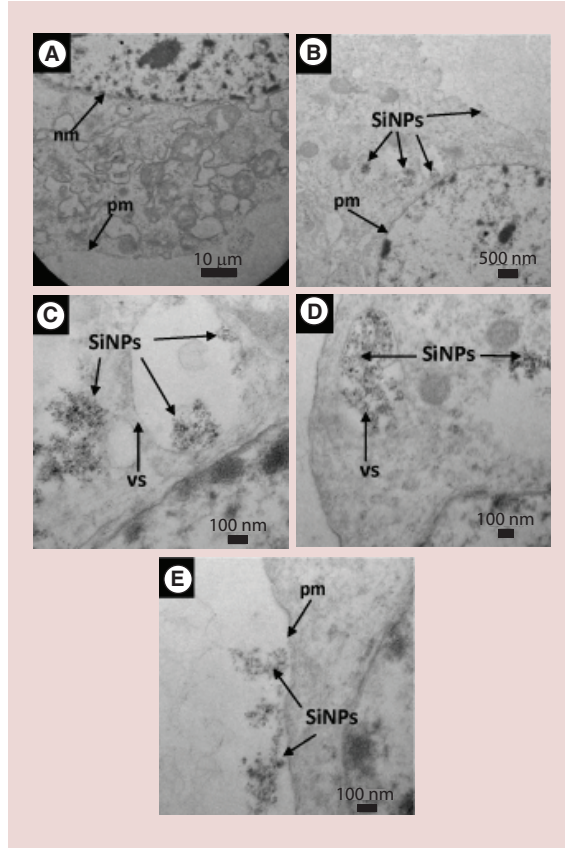

Figure 6. Transmission electron microscopy images of interactions of silica nanoparticles (10 nm) with primary human umbilical endothelial cells. (A) Control untreated cells; **(B–E)** SiNPs-treated cells. SiNPs were found inside endothelial cells – in the cytoplasm or inside the vs. Some SiNPs were in association with the pm and the nm.
nm: Nuclear membrane; pm: Plasma membrane; SiNP: Silica nanoparticle; vs: vesicle.

Diesel exhaust particles (DEP) represent a serious environmental concern and it has been shown that they decrease the bioactivity of vascular NO and enhance the vulnerability of atherosclerotic plaques [109,110]. Moreover, Solomon *et al.* [111] have shown that DEP were internalized by platelets, and induced concentration-dependent platelet aggregation *in vitro*, whereas carbon black (CB) NPs did not, even though they were also internalized. Though CB NPs did not cause platelet aggregation, Vesterdal *et al.* [112] have shown that CB NPs generate oxidative stress associated with vasomotor dysfunction in cultured EC and artery segments. The CB exposure was associated with increased surface expression of ICAM-1 and VCAM-1 in human umbilical vein ECs.

Interestingly, yet another allotrope of carbon, nanodiamond may play a role in nanoimaging and nanotherapeutics. Kumari *et al.* [113] examined platelet compatibility of nanodiamond (4–10 nm) and found

that when administered intravenously in mice, nanodiamonds induce rapid platelet activation (by leading to an increase in intracellular free calcium and the content of platelet tyrosine phosphoproteome), and lead to widespread pulmonary thromboembolism. Nanodiamonds brought about also extensive activator changes in platelets, as revealed by enhanced AnxA5 binding to platelet surface membrane, disruption of the mitochondrial transmembrane potential and generation of reactive oxygen species.

Silver NPs

The antimicrobial activity of silver NPs is extensively used in medicine and food industry [114]. However, whether nanosilver exerts desirable or undesirable effects on vascular hemostasis remains controversial. Jun *et al.* [115] have shown that silver NPs increase platelet aggregation, as well as procoagulant activity *in vitro* and *in vivo* following intratracheal administration in rats. Similarly to surface-bound carbon nanotubes, an antimicrobial composite coating consisting of a pyridinium polymer and silver bromide NPs induced platelet activation upon contact with the coating [116]. These data are indicative of vascular toxicity.

By contrast, Smock *et al.* [117] conducted a placebo-controlled, single-blind, dose-monitored, cross-over study in 18 healthy human volunteers. After 2 weeks of daily oral colloidal silver NP (32 ppm) ingestion, platelet aggregation was evaluated *ex vivo* by light transmission aggregometry in response to collagen and ADP agonists. The results did not support enhanced human platelet activation at low silver serum concentrations (<10 μg/l) resulting from oral ingestion of silver NPs. However, it is worth to point out that light aggregometry may not be sensitive enough for studying NP-induced platelet aggregation that often leads to formation of platelet microaggregates that are below the detection limit of this method [43].

Krajewski *et al.* [118] evaluated the hemocompatibility of whole human blood with different silver NP concentrations (1, 3 and 30 mg/l) employing a modified Chandler-loop *in vitro* assay. The first two concentrations were found to be hemocompatible. Only 30 mg/l induced hemolysis of erythrocytes, caused α-granule secretion in platelets and activated the coagulation cascade.

Other researchers highlighted also potential desirable effects of nanosilver. Shrivastava *et al.* [119] characterized the antiplatelet properties of silver NPs and found that they target platelet granules and exert antiplatelet effects via inhibition of integrin-mediated platelet responses both *in vitro* and *in vivo*. This raised a possibility that nanosilver *per se* could be used as an antiplatelet agent. Furthermore, Ragaseema *et al.* [120] investigated the antithrombotic and antimicrobial properties of PEG-protected silver NP-coated surfaces. They found that these NPs inhibited surface-induced platelet adhesion and aggregation and also exerted bactericidal action. The authors suggested that PEG-nanosilver-coated surfaces may be used in the manufacturing of vascular stents.

Thus, more studies are required to compile the pharmacological/toxicological profile of nanosilver.

Gold NPs

Injectable gold salts comprising of submicron gold have been used for many years in the treatment of rheumatoid arthritis; however, this treatment is associated with severe side effects and toxic effects [121]. Gold NPs are now regarded as promising tools for photo-based diagnostic and therapeutic applications [122].

Love *et al.* [123] examined the effects of gold NPs (30 nm), with positively and negatively charged surface coatings on human blood, showing low reactive oxygen species generation, and the lack of detectable platelet aggregation upon short-term exposure. However, Deb *et al.* [124] found that the interactions of gold NPs with platelets may increase with the decrease of NP size. Using QCM-D to measure platelet activation we found that bare gold NPs 15 nm in size increase platelet aggregate formation, an effect prevented by PEGylation [125].

Polystyrene NPs

As previously indicated, the physicochemical properties of NPs can also determine their biological reactivity. McGuinnes *et al.* [126] investigated how NP surface chemistry impacts the bioactivity of NPs in a platelet activation model. The authors used polystyrene latex NPs of the same size but with different surface charge – unmodified, aminated and carboxylated. Both carboxylated and aminated NPs caused platelet aggregation, whereas the unmodified did not. However, when investigated using the QCM-D [43] and under flow conditions, the unmodified polystyrene NPs led to platelet aggregation. Thus, surface chemistry may affect NP-induced platelet aggregation. Furthermore, Mayer *et al.* [127] confirmed that NP size (also in the case of polystyrene NPs) is inversely correlated with the potential to induce platelet aggregation.

Magnetic & semiconductor NPs

Magnetic nanomaterials including iron oxide NPs are becoming increasingly important in cancer diagnostics and therapeutics [128].

Hermann *et al.* [129] have investigated the effects of combustion-derived carbon-coated iron core/shell nanomagnets (ultrastrong metal nanomagnets; three

times higher in magnetization compared with oxide NPs) on platelet-EC function. They have found that these nanomagnets at concentrations ≤1 mg/ml did not significantly affect platelet-EC function. However, high concentrations of magnetic NPs can cause endothelial damage as indicated by Su *et al.* [130].

Semiconductor NPs such as cadmium QDs can be used in fluorescence imaging to visualize the chosen target. Dunpall *et al.* [131] analyzed the effects of cadmium-selenium QDs capped with cysteine on platelets and found that these NPs concentration-dependently increase platelet aggregation.

Thus, both magnetic and semiconductor NPs can potentially cause vascular toxicity.

Dendrimers

Structural perfection of dendrimers such as poly (amidoamine), PAMAM with numerous functional groups makes them attractive candidates for drug delivery systems. Jones *et al.* [132] tested G7 PAMAM dendrimers and found that cationic dendrimers activate platelets and significantly alter their morphology. These changes to platelet morphology and activation state substantially altered platelet function, including increased aggregation and adhesion to surfaces. Surprisingly, dendrimer exposure also attenuated platelet-dependent thrombin generation which could attenuate prothrombotic properties of these nanomaterials. The same group followed the previous report, with a study [133] demonstrating that amine-terminated dendrimers act directly on fibrinogen in a thrombin-independent manner and generate fibrinogen aggregates. This behavior is most probably mediated by electrostatic interactions between the densely charged cationic dendrimer surface and negatively charged fibrinogen domains, which was further confirmed by the fact that the same dendrimers also aggregate other negatively charged blood proteins such as albumin.

Dobrovolskaia *et al.* [134] explored the subject of PAMAM dendrimers impact on platelets even further by evaluating 12 PAMAM dendrimer formulations of varying size and surface charge. They found that only large cationic dendrimers, but not anionic, neutral or small cationic dendrimers, induce aggregation of human platelets *in vitro*. The aggregation caused by large cationic dendrimers was proportional to the number of surface amines, and probably caused by disruption of platelet membrane integrity.

Biologically derived NPs

In physiology, human body contains nanosized materials such as lipid/protein and protein/mineral complexes that contribute to regulation of vascular homeostasis. Our body is also exposed to nanosized pathogens including viruses, prions and bacterias and these infections may lead to thrombotic/hemorrhagic diseases. Interestingly, nanosized vesicles derived from pathogens and/or activated human cells can calcify and modify platelet function, thus contributing to the pathogenesis of vascular diseases [135,136].

Table 3 summarizes the desirable and undesirable effects of selected NPs on platelets.

Undesirable & toxic effects of nanomaterials on ECs

Apart from blood platelets, NPs can exert undesirable and toxic effects on ECs. Rabolli *et al.* [137] found that the deleterious effects of amorphous silica NPs on ECs are strongly dependent on the physico-chemical characteristics of the NPs including size, surface area, and microporosity.

Zhu *et al.* [138] investigated the influence of iron oxide NPs on human aortic ECs and shown that ECs exposed to two types [Fe(II) and Fe(III)] of iron oxide NPs underwent cytoplasmic vacuolation, mitochondrial swelling and cell death, combined with a significant increase in NO production. Additionally, enhanced monocyte adhesion to ECs was noted, as a consequence of ICAM-1 and IL-8 upregulation and these reactions are considered to be the first steps in atherosclerosis.

Danielsen *et al.* [139] evaluated the impact of several types of titanium oxide, zinc oxide and silver NPs on ECs. Zinc and silver NPs were found to be cytotoxic toward ECs, while titanium oxide led to an increase in expression of ICAM-1 and VCAM-1 in ECs, with the anatase form being more potent than the rutile form, and larger titanium oxide NPs being more cytotoxic than smaller ones. Additionally, silver NPs caused increased monocyte adhesion to ECs.

Smulders *et al.* [140] investigated the impact of titanium oxide, silver and silica oxide NPs, which are found in pristine and aged paints, on a co-culture model of the lung–blood barrier (including microvascular ECs), and found that only NPs from pristine paints, at relatively high concentrations, exerted a cytotoxic effect on the cells, while NPs from aged paints did not. Mikkelsen *et al.* [141] also analyzed the effect of NPs found in paints (three forms of titanium oxide, aluminium silicate, carbon black, nanosilicasol or oxilate). The most significant finding was that primary NPs caused increased cell surface expressions of VCAM-1 and ICAM-1.

Sisler *et al.* [142] investigated the effect of printer-emitted engineered nanoparticles (PEPs) on human microvascular ECs in an alveolar-capillary co-culture model. The authors found that even low concentra-

Table 3. Desirable and undesirable effects of selected nanoparticles in relation to platelet function.

Nanoparticle type	Desirable effects	Undesirable effects
Biologically derived	–	Calcify and modify platelet function [135,136]
Chitosan	Platelet compatible <10 μg/ml [77]	–
Carbon (single- and multi-wall nanotubes, mixed)	–	Stimulate platelet activation aggregation and increase the rate of vascular thrombosis [105–108]
Carbon black	–	Generate oxidative stress associated with vasomotor dysfunction in cultured ECs and artery segments [112]
Dendrimers	Drug delivery systems [83]	G7 PAMAM activate platelets and increase their aggregation and adhesion [132]
	Molecular imaging of platelet activation [56]	
	Attenuates platelet-dependent thrombin generation [132]	
Diesel exhaust particles	–	Decrease bioactivity of vascular NO. Enhance the vulnerability of atherosclerotic plaques [109,110]
		Internalized by platelets – induce concentration-dependent platelet aggregation *in vitro* [111]
Gold	Photo-based diagnostics and therapeutics [122]	Bare cause platelet aggregation [125]
	When PEGylated do not cause platelet aggregation [125]	
Liposomes-cyclic RGD conjugated	Successfully target GPIIb/IIIa [78]	–
Magnetic	At concentrations ≤1 mg/ml do not significantly affect platelet-EC function [129]	At high concentrations can inhibit EC proliferation [130]
	Used to visualize intraluminal thrombi, and to enhance MRI sensitivity [47–55]	Cadmium QDs increase platelet aggregation [131]
PLGA	Platelet compatible <10 μg/ml [77]	–
	Microparticles can target specific vascular sites with high shear-stress to release thrombolytic drugs [88]	
	Half-life resembling that of a biological platelet [75]	
PLGA-chitosan	Platelet compatible <10 μg/ml [77]	–
PLGA-glycocalicin conjugated	Strong adhesion to P-selectin- and vWF-coated surfaces [81]	–
	Increased uptake by activated ECs under shear stress [81]	

EC: Endothelial cell; NO: Nitric oxide; PLGA: Poly(L-lactic-co-glycolic acid); PLL: Poly-L-lysine; QD: Quantum dot; RGD: Arg-Gly-Asp.

Table 3. Desirable and undesirable effects of selected nanoparticles in relation to platelet function (cont.).

Nanoparticle type	Desirable effects	Undesirable effects
PLGA-PEG-GPIb conjugated	Suppress neointimal stenosis and inhibit EC inflammation [80]	–
PLGA-PLL-RGD nanospheres	*In vitro* differentiate activated from nonactivated platelets [74] *In vivo* interact preferentially with activated platelets and actively arrest bleeding [74]	–
Polystyrene–glycocalicin conjugated	Strong adhesion to P-selectin-coated surfaces [82] Increased uptake by activated ECs under physiological flow conditions [82]	–
Polystyrene, micron-sized, platelet mimicking	Recruitment to platelet aggregates in an *ex vivo* model. High platelet resemblance [73]	Cause platelet aggregation (when aminated or carboxylated) [126], also when unmodified [43] Size inversely correlated with the potential to induce platelet aggregation [127]
Silica	–	Trigger (NO)/($ONOO^-$) [97,98] Activate platelets and stimulate aggregation [97,98] Activate the coagulation cascade [96]
Silver	Hemocompatible up to 3 mg/l [118]. In humans, no platelet activation at low silver serum concentrations (<10 μg/l) [117] Antimicrobial effects [114] Antiplatelet effects via inhibition of integrin-mediated platelet responses both *in vitro* and *in vivo* [119]	Increase platelet aggregation and procoagulant activity both *in vitro* and *in vivo* in rats [115]
Silver-PEG	–	Inhibit surface-induced platelet adhesion and aggregation and exert bactericidal effects [119]
Nanodiamond	–	Induce platelet activation and lead to pulmonary thromboembolism *in vivo* in mice [113]

EC: Endothelial cell; NO: Nitric oxide; PLGA: Poly(L-lactic-co-glycolic acid); PLL: Poly-L-lysine; QD: Quantum dot; RGD: Arg-Gly-Asp.

tions of PEPs (0.5 and 1.0 μg/ml) caused morphological changes of actin remodeling, gap formations within the EC monolayer, increased production of reactive oxygen species and angiogenesis. This shows that PEPs at low, noncytotoxic exposure levels are bioactive and affect cellular responses.

Strobel *et al.* [143] investigated cerium dioxide and silicon dioxide effects on ECs and concluded that any alterations on the metabolic cell activity becomes visible at NP concentrations that are by far higher than those expected to occur in *in vivo* situations. The same group [144] came to similar conclusions when investigating the effect of titanium oxide NPs of varying sizes (from 17 to 87 nm) on ECs.

Additional information regarding unwanted pro- and anticoagulant properties of NPs on the coagulation system can be found in the review by Ilinskaya and Dobrovolskaia [145].

Proposed mechanisms involved in nanomaterial–platelet–EC interactions

Currently, the exact mechanisms by which NPs induce platelet activation and aggregation are not well known. Figure 7 depicts mechanisms, proposed by us, that potentially could be involved in nanomaterial–platelet–EC interactions. NP-induced platelet aggregation is most probably mediated by changes in intraplatelet calcium levels [111,115]. Whether this occurs through stimulation of platelet receptors [106], NP endocytosis and subsequent introduction to the canalicular system [98,124,146] or direct physical breach of the platelet membrane and intraplatelet calcium stores remains to be determined. Other pathways leading to NP-induced platelet aggregation may include receptor conformational changes, for example, the GPIIb/IIIa, related to direct physical contact with the NP, 'bridging' of nonactivated platelets by NPs [146], and 'flip-flopping' of the platelet membrane (leading to PS exposure) [115] as a result of NPs colliding with the platelet and activating the coagulation cascade (Figure 7). It has also been shown that NPs, at concentrations insufficient to cause platelet aggregation, can synergize with subthreshold doses of physiological agonists, such as ADP or TXA_2, to trigger full platelet response [106].

NPs increase the potential for platelet adhesion and aggregation also through their interactions with the vascular endothelium. When interacting with ECs, NPs can lead to an increased expression of VCAM-1 and ICAM-1 [112] and also cause enhanced O_2^- radical generation leading to generation of $ONOO^-$ [98]. The latter effect leads to subsequent cellular death and exposure of the subcellular matrix triggering platelet activation.

Conclusion

In this review article we have focused on the nanodiagnostics, nanopharmacology and nanotoxicology of platelet–vessel wall interactions. A relatively broad selection of devices is available to investigate the interaction occurring between blood platelets and the vessel wall, with remodelable and endothelialized microfluidic devices, as well as the QCM-D emerging as the most promising ones.

Nanodiagnostics of platelet–vessel wall interactions chiefly focuses on developing contrast-enhancing NPs, such as conjugated or nonconjugated magnetic iron oxide NPs of different sizes. The *in vivo* use of these NPs enhances MRI visualization of intravascular atherosclerosis-associated inflammatory changes.

Platelet-mimicking NPs emerge as a potential therapeutic option to arrest major trauma or surgery-induced bleeding, as well as treat patients with platelet-related bleeding disorders. Studies also show that NPs can be successfully used as drug nanodelivery systems, especially when it comes to administering thrombolytic therapy. Most NPs tested as potential drug delivery systems appear to be blood compatible.

However, potential side effects of NPs in platelet–vessel wall interactions need to be also considered. Once in the bloodstream NPs can directly interact with platelets and ECs changing their properties and platelet-EC interactions. The $(NO)/(ONOO^-)$ imbalance may trigger off undesirable effects of nanomaterials leading to EC hemostatic and vasomotor dysfunction and vascular thrombosis (e.g., silica and carbon NPs, nanodiamonds, dendrimers, DEP).

One should carefully weigh both the desirable and undesirable effects of NPs before deciding to use them clinically. Undeniably NPs will play a major part in future nanodiagnostics and nanotherapeutics.

Future perspective

It is clear that the dynamic growth of nanoscience and nanotechnology has had a significant impact on cardiovascular medicine. In future we envisage further developments in nanodiagnostics and nanotherapeutics concerning the hemostatic/thrombotic balance.

Given wide-spread use of antiplatelet drugs and a relative paucity of easy-to-use, inexpensive platelet function measuring devices, the development of point-of-care nanodevices could offer new standards in personalized medical care of patients with occlusive vascular diseases. Iron oxide and other magnetic NPs may provide unique thrombus-selective live imaging and diagnostics, and when combined with an antiplatelet drug-releasing function, pharmacological opportunities. Currently, imaging of individual

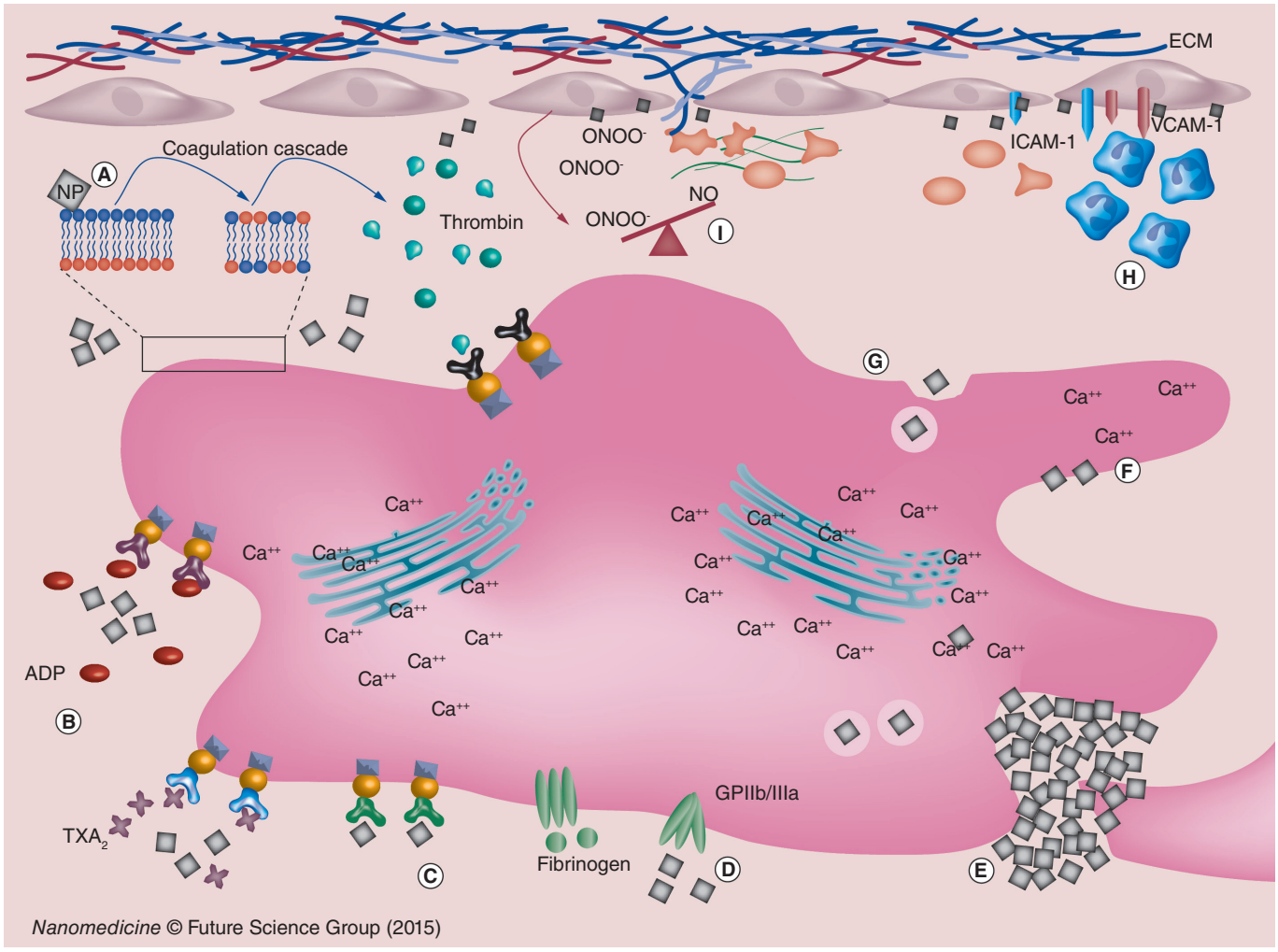

Figure 7. Proposed mechanisms involved in nanomaterial–platelet–endothelial cell interactions: role of Ca^{++}. **(A)** Nanoparticle collision with platelet membrane leading to membrane 'flip-flop' and exposure of phosphatydyloserine, and subsequent activation of the coagulation cascade. **(B)** Nanoparticles synergize with physiological agonists (e.g. ADP or TXA_2). **(C)** Direct stimulation of platelet receptors by nanoparticles. **(D)** Nanoparticles directly induce conformational changes of platelet receptors. **(E)** Bridging of nonactivated platelets by nanoparticles. **(F)** Direct physical breach of the platelet membrane by nanoparticles and release of intraplatelet Ca^{++} stores. **(G)** Nanoparticle endocytosis and trafficking to the dense canalicular system, leading to the release of intraplatelet Ca^{++} stores. **(H)** Nanoparticles increase expression of VCAM-1 and ICAM-1. **(I)** Nanoparticles enhance generation of $ONOO^-$.
DTS: Dense tubular system; ECM: Extracellular matrix; NO: Nitric oxide; $ONOO^-$: Peroxynitrite.

platelet–vessel wall interactions is not possible due to limits in spatial resolution and/or restricted penetration depths of noninvasive imaging modalities. However, this limit will surely soon be overcome as the current invasive methods have an imaging resolution of ~10 µm. Such an advance will allow for real-time, *in vivo* monitoring of individual platelet–vessel wall interactions.

Progressing from development of platelet-mimicking NPs to NP-engineered synthetic platelets; fully resembling physiological platelets could attenuate the need for transfusions with platelet concentrates. This will have a significant impact on the treatment of acute injuries resulting in massive bleeding, as well as on the treatment of inherited and acquired platelet defects. Finally, understanding physiological and pathological roles of biologically derived NPs may be of significant importance for cardiovascular medicine.

All the existing and future technological and pharmaceutical developments need to be carefully balanced against the inherent risk of toxicity associated with nanomaterials. However, future research is likely to bring about new ways of reducing NPs toxicity by modifying NP size, charge and surface or using non-biological nanomaterials (likely to persist in the body for a long time) only as templates for forming nontoxic, biodegradable particles. This is of particular impor-

tance as the treatment of vascular conditions associated with vascular dysfunction is not only therapeutic when it comes to the management of the primary vascular event, but also a preventive one to protect patients from secondary events. Another approach might concentrate on finding ways of increasing NP metabolism and excretion – either through the respiratory, urinary or gastrointestinal tract. Preventing NP accumulation may considerably limit their deleterious effects on the living organism.

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Executive summary

Platelets & the vascular endothelium

- A systematic search of the literature was conducted to review state-of-the-art of nanomaterial–platelet–endothelial cell interactions.
- Platelet–endothelial cell interactions play a crucial role in vascular hemostasis supporting vascular integrity.
- A deficient regulation of these interactions leads to thrombosis that complicates the clinical course of vascular diseases.

Diagnostics of platelet activation: role of micro- & nano-fluidic devices for studying platelet vessel wall interactions

- Platelet function can be measured in a very sensitive way using microfluidic and nanofluidic devices.

Nanodiagnostics of platelet–vessel wall interactions

- Molecular and whole cell nanoimaging can be used to study platelet–endothelial cell interactions.

Desirable effects of nanomaterials in platelet–vessel wall interactions

- Nanomaterials exert desirable and undesirable effects in platelet–vessel wall interactions.
- Platelet-mimicking nanoparticles and nanocarrier-containing thrombolytic drugs exemplify desirable effects.

Undesirable & toxic effects of nanomaterials in platelet–vessel wall interactions

- Undesirable effects have been demonstrated for a number of nanoparticle families including carbon, silica, magnetic and semiconductor nanoparticles.
- Endogenous calcifying nanoparticles may play a role in the pathogenesis of vascular diseases.
- The generation of oxygen- and nitrogen-reactive species may play a major role in the pathomechanism of nanoparticle toxicity.

Proposed mechanisms involved in nanomaterial–platelet–endothelial cell interactions

- The mechanisms through which nanoparticles influence platelet–vessel wall interactions are complex and may involve calcium-dependant reactions.
- The surface-volume ratio, zeta potential as well as other physicochemical properties of nanomaterials may contribute to their mechanisms of cellular action.
- Understanding desirable and undesirable effects of nanoparticles in platelet–vessel wall interactions is crucial for the development of advanced strategies in diagnostics and therapeutics of vascular diseases.

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