HHS Public Access

Author manuscript

Platelets. Author manuscript; available in PMC 2023 January 02.

Published in final edited form as:

Platelets. 2022 January 02; 33(1): 35–47. doi:10.1080/09537104.2021.1967916.

Bioinspired Artificial Platelets: Past, Present and Future

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Abstract

Platelets are anucleate blood cells produced from megakaryocytes predominantly in the bone marrow and released in blood circulation at a healthy count of 150,000-400,00 per μL and circulation lifespan of 7-9 days. Platelets are the first responders at the site of vascular injury and bleeding, and participate in clot formation via injury site-specific primary mechanisms of adhesion, activation and aggregation to form a platelet plug, as well as secondary mechanisms of augmenting coagulation via thrombin amplification and fibrin generation. Platelets also secrete various granule contents that enhance these mechanisms for clot growth and stability. The resultant clot seals the injury site to stanch bleeding, a process termed as hemostasis. Due to this critical role, a reduction in platelet count or dysregulation in platelet function is associated with bleeding risks and hemorrhagic complications. These scenarios are often treated by prophylactic or emergency transfusion of platelets. However, platelet transfusions face significant challenges due to limited donor availability, difficult portability and storage, high bacterial contamination risks, and very short shelf life (~ 5-7 days). These are currently being addressed by a robust volume of research involving reduced temperature storage and pathogen reduction processes on donor platelets to improve shelf-life and reduce contamination, as well as bioreactor-based approaches to generate donor-independent platelets from stem cells in vitro. In parallel, a complementary research field has emerged that involves the design of *artificial platelets* utilizing biosynthetic particle constructs that functionally emulate various hemostatic mechanisms of platelets. Here we provide a comprehensive review of the history and the current state-of-art of artificial platelet approaches, along with discussing the translational opportunities and challenges.

Platelet transfusion in the clinic

Platelets are anucleate discoid 2-4 μm diameter blood cells produced from megakaryocytes predominantly in the bone marrow and released in blood circulation (1,2). In a healthy individual, the normal platelet count in blood is 150,000-400,00 per μL, with a circulation lifespan of 7-9 days. The main physiological role of platelets is to render hemostasis (stoppage of bleeding) in the event of blood vessel injury, by *primary* mechanisms of

Contributions

All authors contributed equally to the writing of the manuscript.

injury site-specific adhesion (via platelet GPIbα binding to VWF, and platelet GPVI and GPIa/IIa binding to collagen), activation (via specific *inside-out* and *outside-in* signaling) and aggregation (via fibrinogen binding to platelet surface integrin GPIIb-IIIa) to form the hemostatic platelet plug (3-15). A subset of injury site-localized platelets also facilitates *secondary* mechanisms of augmenting coagulation outputs via amplifying thrombin generation on anionic phospholipid-rich active platelet surface and secreting various procoagulant biomolecules from platelet granules (16-20). These central mechanisms of platelet role in hemostasis are shown in Figure 1.

Due to this critical role, a reduction in platelet number (due to decreased production, increased consumption or rapid depletion) or dysregulation in platelet function (congenital, pathology-associated or drug-induced) is associated with bleeding risks and hemorrhagic complications. Clinical manifestations of such scenarios are exemplified in congenital or drug-induced or immune thrombocytopenia (reduced platelet count), Bernard-Soulier syndrome (dysfunction in platelet GPIbα binding to vWF), Glanzmann's Thrombasthenia (dysfunction in platelet GPIIb-IIIa binding to fibrinogen), anti-platelet therapy effects in complex surgical settings (reduced platelet response), and traumatic hemorrhage (rapid depletion and trauma-induced dysfunction). These clinical scenarios are often treated by prophylactic (to reduce bleeding risk) or emergency (to mitigate active bleeding) transfusion of platelets (21-28). The platelet units for transfusion are obtained by pooled platelets derived from whole blood of multiple donors or apheresis-based platelet isolation from single donor, with approximately 2-3 x 10^{11} platelets administered per transfusion unit. Starting from the first demonstration of successful platelet transfusion by Murphy and Gardner in 1969, current clinical practice for donor-derived platelets is to store them at room temperature (RT, 20°C-24°C) with gentle agitation (29, 30). Over the past several decades a robust volume of studies has provided significant evidence regarding the benefits of platelet transfusion in treating bleeding risks and hemorrhagic complications, however, platelet transfusion products present several persistent logistical and functional challenges. One persistent logistical issue is the constant shortage of blood donors, which severely impacts platelet availability (31-34). Other significant issues are the very short shelf-life (~5 days) and high risks of pathogen contamination of RT platelets (35, 36). Additionally, platelet transfusion infrastructure is limited to large hospitals and trauma centers, and therefore not broadly available at all medical locations. Furthermore, platelet transfusions are not available at all in pre-hospital settings to treat actively hemorrhaging patients (e.g. in traumatic injuries and mass casualty events), and therefore the distance between the point-of-injury and the nearest transfusion center has a significant effect on the timely transfusion and patient survival (37). The lack of pre-hospital availability for trauma resuscitation is a major barrier. Robust data support that moving blood component resuscitation with packed red blood cells and plasma into the field improve outcomes after injury (38-40). Platelets remain the "final frontier" of blood component transfusion in the pre-hospital setting, and data supporting improved outcomes with early platelet transfusion in massive hemorrhage strongly support the rationale to move platelet transfusion forward along with PRBCs and plasma (28). Other specific functional challenges include platelet refractoriness as well as platelet-induced harmful immune or inflammatory responses in subsets of patients(41, 42).

To address the above challenges with platelet transfusion, several parallel research efforts are being conducted. For example, extensive efforts are being directed at reducing contamination risks in platelets using pathogen reduction technologies (PRT) (35, 36). Bacterial contamination of platelets occurs at a frequency of 1 in every 1000-2500 units, and can lead to transfusion-transmitted bacterial infections (TTBI) and septic transfusion reactions (STR) in patients. PRT approaches to reduce such risks predominantly utilize ultraviolet (UV) light based systems, e.g. the INTERCEPT™ system that uses UV irradiation of photosensitive compound *amotosalen* added to platelet units for broad spectrum pathogen inactivation. Post-PRT, the residual amotosalen and photoproducts need to be removed via a special adsorption device within 24 hours of platelet collection. Another PRT approach is Mirasol® that uses UV-irradiation of *riboflavin* as the photosensitive reactive oxygen species (ROS) producer, to inactivate bacteria, virus and parasites. Yet another PRT approach in development is THERAFLEX® that uses short wave UV irradiation of platelet bags to affect nucleic acid strands and thus inactivate bacteria and virus. These various PRT processes add to the cost of platelet transfusion logistics, and while they enhance the safety of platelet transfusion products, their effect on platelet count, circulation lifespan and function *in vivo* remains an area of continued research.

An important strategic direction to increase platelet shelf-life involves processing of platelets at reduced temperature (e.g. freeze-drying platelets), as well as storing platelets at low temperatures (e.g. cold-stored and cryopreserved platelets) (42-50). Freeze-dried platelets involve lyophilizing platelets in presence of protein crosslinkers (e.g. paraformaldehyde) or lyoprotectants (e.g. Trehalose) to yield a room-temperature storable aqueous reconstitutable powder, cold-stored platelets involve storage of platelet concentrate at 4°C, and cryopreservation involves freezing to −80°C in presence of cryoprotectant (e.g. dimethyl sulphoxide, DMSO) and then storing at −65°C or lower. Reports on cooling or lyophilizing platelets for transfusion date back to the 1950s and cryopreservation was reported in the 1970s, but their widespread use in transfusion medicine has not been adopted broadly yet. This is partly due to their rapid clearance from circulation by hepatic macrophages (circulation lifespan hours to ~ 1.5 days) compared to RT platelets (life-span ~ 4 days) (47, 48). Reduced temperature processing (as well as storage) of platelets also induce certain functional changes in platelets, including desialylation, GPIbα clustering, partial loss of GPIIb-IIIa function, increased activation leading to thromboxane A2 production, procoagulant phosphatidylserine (PS) exposure and formation of a higher percentage of PS-positive microparticles, collectively termed ‘cold storage lesion’, which accelerate the macrophagic clearance of such platelets and render them sub-optimal for prophylactic transfusion (e.g. in thrombocytopenia) (51- 54). At the same time, there is emerging recognition that such functional changes may make these platelets *hemostatically primed* for rapid clot formation and thereby provide emergency therapeutic benefit in the hemostatic management of actively hemorrhaging patients (e.g. in surgery, trauma, acute radiation poisoning etc.). This has resulted in renewed interest for evaluating such platelet products for therapeutic transfusion in civilian and military scenarios (55-59). In relevant context, the use of cold-stored low-titer group O whole blood (LTOWB) is growing as a result of documented safety and efficacy in trauma centers (60-62). Since LTOWB contains platelets, therefore the rapidly evolving practice of transfusing cold-stored

LTOWB suggest hemostatic ability of cold platelet transfusion, but further studies focused specifically on cold-stored platelets are needed (63, 64). To this end, a clinical trial termed 'Chilled Platelet Study' (CHIPS) has been recently initiated in the US (Trial Identifier: NCT04834414). There is also some renewed interest in *temperature cycling* of stored platelets where it has been demonstrated that instead of storing platelets continuously at RT or at 4°C, warming cold platelets to RT for small time-intervals every 12 hrs may reduce the extent of 'cold storage lesion', but these strategies would be cost- and energy-intensive for practical purposes. An additional strategic and exciting direction involves potentially increasing platelet availability via production of platelets and platelet-like cells *in vitro* from precursor cells (e.g. megakaryocytes, stem cells etc.) using unique bioreactor designs (65-69). Excitingly, such *in vitro* platelet generation is also enabling the use of CRISPR/Cas9 technology to create human leukocyte antigen (HLA)-universal platelets which may avoid immune reaction and transfusion refractoriness (70). However, some challenges still remain for determining megakaryocyte surface markers to ensure cellular differentiation and to reduce the risk of including precursors in transfusions as pluripotent stem cells may engraft and form teratomas (71). Additionally, the current sub-optimal yields of *in vitro* produced platelets limit 'platelet function' assessments by standard assays such as microfluidics, aggregometry, viscoelastometry etc., while flow cytometry-based analyses requires attentive rigor for gating of the metabolically active platelet population (71). Furthermore, the current post-administration half-life of iPSC-derived platelets is only a few hours which is inadequate for prophylactic use, and but their promise warrants continued research to improve circulation lifespan and functions of these platelets (72). While the above research and development efforts are highly promising, none of them are currently envisioned to be a 'stand-alone' exclusive solution that can collectively address all of the persistent challenges in platelet transfusion. Therefore, another alternative strategy, complementary to these efforts, has emerged that involves the development of *artificial platelets* that utilizes biosynthetic particle constructs to functionally recapitulate certain hemostatically relevant mechanisms of platelets. The following sections will provide a comprehensive review of the history and the current state-of-art of such artificial platelet approaches, along with discussing the translational opportunities and challenges.

Natural platelet-dependent *artificial platelet* systems

As described above, the *primary hemostatic* response of platelets involves adhesion (to vWF and collagen) as well as aggregation (mediated by fibrinogen-binding). Therefore, the earliest designs of artificial platelets attempted to emulate these functional outputs by simply extracting platelet membrane glycoproteins (e.g. by detergent method) and incorporating them within the lipid membrane of liposomal vesicles, resulting in a design named 'Plateletsome'(73). An evolved variation of this approach can be identified in the product *infusible platelet membrane* (IPM, Cypress Bioscience), which involved extraction of natural platelet membrane, pasteurization and formation of lyophilized vesicles therefrom, and this product advanced through clinical trials but did not achieve approval (74, 75). Interestingly, in recent years several research groups have reported the utilization of extracted platelet membranes to coat synthetic nanoparticles, demonstrating that some of the bioactive glycoproteins may be conserved on these extracted membranes, to allow

platelet-relevant biointeractions of the coated nanoparticles (76-79). While such membrane extraction-based approaches may allow partial retention of platelet-relevant hemostatically functional surface proteins, the extraction, purification, particle incorporation/interfacing steps and immune compliance aspects required for these approaches potentially make such strategies potentially too complex to scale up, preserve batch-to-batch quality, and advance towards clinical translation through a complicated regulatory pathway. Additionally, these approaches are still dependent on natural platelet membrane functions, which in turn is dependent on platelet availability and biosafety that have their inherent challenges as delineated previously. A promising platelet surrogate technology in this area is Thrombosomes (CellPhire), which is a trehalose-stabilized lyophilized platelet product prepared from apheresis-collected Group O 2-3 day old donor platelets (80-82). This product has shown promising hemostatic capabilities in mitigating bleeding in a variety of animal models, and is currently in clinical evaluation in actively bleeding thrombocytopenic patients. While the *in vivo* circulation lifespan of this lyophilized product may be short (analogous to cold and lyophilized platelet issue stated previously), its emergency use in hemorrhage control can be an exciting option. Figure 2 summarizes the platelet surrogate designs derived from (and dependent on) natural platelets.

Protein-decorated *artificial platelet* systems

Instead of depending on membrane extraction and membrane-derived glycoprotein availability to enable platelet's hemostatic functions, some past approaches focused on using specific natural or recombinant proteins to decorate particle platforms for hemostatic activity. In this area, the earliest approaches involve coating of RBCs as well as synthetic particle platforms with fibrinogen to essentially create 'super-fibrinogen' constructs that may augment the aggregatory kinetics and hemostatic output of activated platelets. Examples of this approach is found in technologies like Synthocytes™, Thrombospheres™ and Fibrinoplate™, all of which are essentially made of human albumin microparticles surface-coated with Fg (83-88). These products have undergone promising preclinical *in vivo* studies and some early phase clinical trials, but have not progressed into rigorous clinical evaluation and translation. The Fibrinoplate™ product was developed by the company Fibroplate Inc. for potential hemostatic applications, however, little information on their current status is available. Another fibrinogen-decorated design that has undergone preclinical and clinical evaluation in the US and Europe is Fibrocaps™ (ProFibrix, The Netherlands), where fibrinogen and thrombin solutions are separately spray-dried and then combined to produce a suspendable microparticle mixture that can enable *in situ* fibrin formation (89). This product needs to be directly applied to the wound (sprayed or applied in a sponge) and therefore is only suitable for topically treating active bleeding from accessible wound sites. Considering the fact that human fibrinogen concentrate itself is clinically approved (e.g. Riastap) for treatment of bleeding, one can envision that fibrinogen-coated particle systems may also provide translationally feasible hemostatic applications. If the fibrinogen is derived from non-human sources (e.g. bovine), potential immunogenic risks need to be adequately mitigated for human use. Also, it is important to note that fibrinogen-decorated particle constructs are not necessarily artificial platelet mimics, but rather they provide a 'clustering' construct to enhance active platelet aggregation. In this aspect, they

may be unsuitable for prophylactic applications (e.g. in thrombocytopenia where platelet numbers are insufficient for hemostatic aggregate formation), but more appropriate for emergency mitigation of certain active bleeding scenarios where fibrinogen may get depleted while platelet numbers may remain sufficient for hemostatic aggregate formation.

While fibrinogen-coated particle constructs have focused on augmenting platelet *aggregation*, other approaches have focused on emulating platelet *adhesion*. In this strategy, the earliest approaches utilized recombinant GPIbα (rGPIbα) to enable binding to vWF and recombinant GPIa-IIa (rGPIa-IIa) to enable binding to collagen. These recombinant proteins were conjugated on the surface of liposomes, latex beads or albumin-based particles (90-93). The resultant constructs were able to effectively adhere to vWF-coated and collagen-coated surfaces simulating hemostatically relevant platelet adhesion *in vitro*. In further advancement of this approach, the rGPIbα and rGPIa-IIa motifs were co-conjugated on the surface of liposomes and albumin particles, and this combination demonstrated higher binding to collagen surfaces in presence of soluble vWF at higher shear rates, closely mimicking natural platelet adhesion (91). It is important to note here that recombinant technology can be quite expensive for clinical translation. Also, the large size of the recombinant protein fragments can cause mutual steric interference regarding their combinatorial decoration on the particle surface. Another promising platelet-inspired design to use protein-decorated approach in recent years has focused on the property of activated platelets to bind fibrin proto-fibrils and enable clot contraction via the modulation of platelet cytoskeletal machinery, which is important for clot stability and wound healing (94). This aspect was emulated via the decoration of polyisopropyl acrylamide based low-crosslinked microgel particles with antibody fragments that bind to fibrin (95). These flexible particles demonstrated platelet-relevant biomechanical aspects of clot contraction and stability, with the caveat that the binding of these constructs would require prior presence of sufficient fibrin (i.e. significant propagation of coagulation) at the injury site. Therefore, hematologic and hemostatic dysfunctions that present sub-optimal coagulation outputs and fibrin generation (e.g. hemophilia, trauma-induced coagulopathy, certain thrombocytopenias etc.) may require additional refinement of this technology for efficient treatment capability. On the other hand, if sufficient fibrin is present, this technology can not only bind to fibrin for platelet-mimetic biomechanical output, but can also act as a carrier platform for other therapeutic agents to allow clot-targeted drug delivery (96, 97). Figure 3 summarizes the artificial platelet designs that utilize particle surface coating with proteins and antibody fragments to emulate various hemostatic mechanisms of platelets.

Peptide-decorated *artificial platelet* systems

Instead of using natural or recombinant proteins or antibody fragments to decorate particle platforms for mimicking hemostatically relevant platelet mechanisms, some approaches have focused on using small molecular weight peptides for analogous functions. To this end, one of the earliest approaches involved surface-decoration of red blood cells (RBCs) with peptides bearing the Arginine-Glycine-Aspartic Acid (RGD) motif, leading to a technology named ‘Thromboerythrocyte’ (98). The design rationale here is similar to coating particles with fibrinogen (described in previous section), since the binding of fibrinogen to platelet surface integrin GPIIb-IIIa is mediated by RGD sequences in

fibrinogen alpha chains (99, 100). The RGD-decorated RBCs were able to co-aggregate with platelets and increased overall aggregation of ADP-activated platelets as measured by light transmission aggregometry. These studies demonstrated the feasibility of using fibrinogen-relevant RGD peptide sequences to decorate particles and thereby create 'super-fibrinogen' constructs to enhance platelet aggregation. This approach has been adapted in recent years by using RGD peptide motifs to decorate poly-lactic acid/poly-glycolic acid based nanoparticle platforms for hemostatic applications (101-103). These approaches have predominantly utilized RGD sequences like CGRGD or GRGDS, that, in spite of their capability to bind platelet GPIIb-IIIa, can have two potential issues in clinical translation: (i) the ubiquitous nature of these RGD motifs to bind to many different integrins on other cells can impart lack of platelet-specificity and risk of cross-reactivity, and (ii) their reported ability to trigger activation of resting platelets can raise systemic pro-thrombotic risks (104-106). Another fibrinogen-relevant peptide motif that has been used to decorate particle systems for hemostatic function is HHLGGAKQAGDV (also known as H-12 peptide) based on sequence present in the γ chain of fibrinogen (107). This peptide is deemed to have higher specificity to platelet GPIIb-IIIa compared to the ubiquitous RGD peptides, and thus H-12-decorated liposomes, latex beads and albumin particles were created to enhance platelet aggregation outputs (108-110). These constructs have all shown promising hemostatic capabilities in vitro in terms of enhancing platelet aggregation. Furthermore, H-12-decorated liposomes were loaded with ADP, and these systems showed promising hemostatic efficacy in rabbit models of thrombocytopenia and hemorrhage (110).

While the RGD- and H-12-decorated particle systems are designed to amplify platelet *aggregation* kinetics for hemostatic output, peptide decorations have also been used to mimic the *adhesion* mechanisms of platelets. An early example of this is found in the use of platelet GPIbα-relevant 15-mer peptides decorated on liposomal particles for potential binding to vWF (111). The biochemical characterizations of these constructs were reported, but evaluation of their actual hemostatic efficacy has not been reported. In a more recent approach, our research has utilized a vWF-binding peptide (VBP) sequence TRYLRIHPQSWVHQI derived from the C2 domain (residues 2303-2332) of the coagulation factor FVIII or a collagen-binding peptide (CBP) sequence of 7-mer repeat of the Glycine(G)-Proline(P)-Hydroxyproline(O) tri-peptide (i.e. -$[GPO]_7$-) with helicogenic affinity to fibrillar collagen but minimal interaction with platelet GPVI, to mimic platelet adhesion mechanisms on liposomal nanoparticles (112, 113). Our studies demonstrated that VBP-decorated particle systems were able to bind vWF-coated surfaces under high shear flow conditions, mimicking platelet's adhesive response to vWF. Additionally, on collagen-coated surfaces, CBP-decorated particle systems were able to adhere at both low and high shear flow conditions. Figure 4 summarizes the artificial platelet designs that utilize particle surface coating with peptides to emulate various hemostatic mechanisms of platelets.

Heteromultivalent decoration: a new direction in designing *artificial platelet* systems

All of the designs described in the above sections involving protein-decorated and peptide-decorated particle constructs focus on a *single* aspect of platelet's hemostatic function: either

adhesion, or aggregation, or fibrin reinforcement. In an ideal hemostatic process, all of these functions need to occur concomitantly (20, 114). This need has provided the rationale to explore unique strategies for combining multiple functionalities either by physically mixing differently decorated particles or by co-conjugating different decorations on the surface of the same particle. In the co-conjugation framework, our studies demonstrated that nanoparticle co-decoration with 'VBP + CBP' enhanced platelet-mimetic adhesion on 'vWF + collagen' -coated surfaces in microfluidic channels at low-to-high shear ranges, compared to nanoparticles bearing VBP decoration only or CBP decoration only (112). The combination decoration approach is termed *heteromultivalent decoration* since multiple types of biofunctional motifs were conjugated on a single particle surface. This design is analogous to particle decoration with 'rGPIbα + rGPIa-IIa' described previously, but with the advantage of utilizing small peptides for decoration which allows higher decoration density on the surface for enhanced synergistic activity without mutual steric interference. While the heteromultivalent decoration of nanoparticles with 'VBP + CBP' motifs still focused on the adhesion property of platelets, an early attempt towards combining 'adhesion + aggregation' was demonstrated in the work of Okamura et. al. by surface-decorating latex beads with a combination of rGPIbα fragment and H-12 peptide motifs, and comparing their hemostatically relevant outputs (surface adhesion and platelet aggregation) *in vitro* to rGPIbα-decorated beads only, H-12-decorated beads only, and a physical mixture of 'rGPIbα-decorated beads + H-12-decorated beads' (115). These studies revealed that if there is a significant size difference between the co-conjugated motifs (e.g. large rGPIbα compared to small H-12 motifs), the synergistic functional output is lower than expected, possibly due to steric interference or masking of bioactivity of the smaller motif by the larger one. From a design perspective, there are two possible ways to resolve this issue: (1) either the motifs need to be presented at different canopy levels from the particle surface by using different spacer molecules to avoid mutual interference, or (2) all decorations need to utilize smaller ligand motifs of extended from the particle surface by the same spacer length. Consequently, in our research we opted for the second strategy by utilizing a combination of vWF-binding peptide (the VBP motif stated previously), collagen-binding peptide (the CBP motif stated previously) and a fibrinogen-mimetic peptide (FMP, with the sequence cyclo-CNPRGDY[-OEt]RC) that has high binding specificity to active platelet GPIIb-IIIa compared to other cellular integrins (116). Our combinatorial decoration of liposomal particles with 'VBP + CBP + FMP' is essentially the very first demonstration of utilizing heteromultivalent peptide decoration to integratively emulate platelet's hemostatically relevant adhesion and aggregation mechanisms on a single particle platform, and our *in vitro* as well as *in vivo* studies demonstrated that this *functional integration* provided higher hemostatic efficacy than particles bearing *adhesion functionality* only ('VBP + CBP'-decoration only) or *aggregation functionality* only (FMP decoration only) (117, 118). This liposome-based artificial platelet technology was named SynthoPlate and this technology has demonstrated promising hemostatic efficacy in mouse thrombocytopenia model, mouse and rat acute liver injury model and pig femoral artery severe hemorrhage model (119-121). This technology could be effectively sterilized and stored as aqueous suspension for up to 9 months without affecting platelet-mimetic bioactivity (121). The SynthoPlate system has been issued several patents and has been recently licensed to a biotechnology company, Haima Therapeutics, for translational development towards clinical

application as an aqueous suspension as well as an on-demand aqueous-reconstitutable lyophilized powder, for potential use in both in-hospital and pe-hospital settings as a platelet surrogate. We have also demonstrated that analogous liposomal particles can be further used as carrier platforms for targeted delivery of additional hemostatic agents (e.g. Tranexamic Acid or TXA, an antifibrinolytic agent) to enhance hemostatic efficacy while maintaining systemic safety (122). The *heteromultivalent* surface-decoration strategy is not limited only to liposomal particles, but can be adapted to other material platforms utilizing appropriate bioconjugation chemistry, as we have demonstrated by conjugating the peptides to albumin, hyaluronic acid etc. (123, 124). The additional envisioned benefit of SynthoPlate is that the peptides as well as the liposomal particle platform are fully synthetic, non-immunogenic, biocompatible and biodegradable components and thus the *in vivo* application does not require any type matching (i.e. universally transfusable). Figure 5 summarizes the artificial platelet designs that utilize heteromultivalent surface decoration of particles to integrate adhesive and aggregatory mechanisms of platelets in hemostasis.

Design evolution and future directions with *artificial platelet* systems

The current state of basic research and pre-clinical development of RGD or H-12 peptide-decorated particle systems, fibrin-binding particle systems and heteromultivalently decorated particle systems, along with demonstration of *targeted payload delivery* with such particles as carrier platforms, provide promising evidence on how nanoscale molecular engineering can be employed to mimic various hemostatic mechanisms and functions of platelets. Table 1 summarizes the various ‘platelet surrogate’ approaches, along with their stage of development and representative evaluation status. It is important to note that the *artificial platelets* terminology does not imply that the biomimetic constructs comprehensively emulate all the biological complexities of platelet mechanisms, signaling and function (125), but rather they modularly emulate specific platelet-relevant mechanisms or functions in appropriate applications (platelet-*inspired* modular design rather than platelet-*equivalent* comprehensive design). That said, some research approaches are also focusing on synthetic biology tools to attempt the mimicry of more complex platelet mechanisms in phospholipid vesicles (126). Recently we have also successfully demonstrated the possibility of incorporating other platelet-relevant hemostatic components like anionic phospholipids and inorganic polyphosphates for procoagulant function within artificial platelet design, to emphasize the customizability of such constructs for both primary and secondary hemostatic aspects of platelet function (127). Another interesting area of research is the mimicry of platelet’s biophysical aspects, e.g. size, shape and stiffness, which influence their fluid dynamic and margination behavior *in vivo* and thereby influence their hemostatic responses (128). The advent of bottom up and top-down manufacturing methodologies to control particle geometry, morphology and modulus, has opened the door to explore the potential incorporation of such *biophysical* design parameters into enhancing the performance of artificial platelet constructs (123, 129-131). Additional research endeavors involve exploring the integration of platelet-mimetic systems with RBC-mimetic systems and plasma to potentially create biosynthetic whole blood surrogate formulations (132). The possibility of such approaches were recently demonstrated by combining H-12 peptide-decorated, ADP-loaded liposomes (hemostatic particles) with hemoglobin-loaded liposomes (oxygen

transporting particles) in treating traumatic hemorrhage in thrombocytopenic rabbits (133). Beyond thrombocytopenia and trauma, artificial platelet systems need to be further evaluated in treating other hematologic dysfunctions, as well as in thrombosis, immune response, inflammation and cancer pathologies, where significant opportunities remain for designing platelet-inspired constructs towards translational therapeutic applications (134, 135).

In translational advancement of *bioinspired artificial platelet* technologies, several important parameters and risk factors will need to be further assessed, including batch scalability and cost-effective manufacturing, recurrent and escalating dose effects, potential systemic thrombosis and coagulation risks at various doses, and potential immunogenic risks. For example, the studies reported so far regarding the various artificial platelet systems, including our own research, have mostly used a single dose and a single administration (by infusion or bolus) *in vivo*. While these have shown promising efficacy, significant opportunities remain in advancing the technologies to enable safety and efficacy of repeat dose and dose escalation parameters, that may be important in treating acute or chronic bleeding dysfunctions. Regarding immunogenicity, it is well established that compared to larger protein structures, small peptides have much reduced immunogenicity and this, in fact, has remained a challenge in creating peptide-based vaccines (136). Therefore, peptide-decorated nanoparticle designs of artificial platelet may, by default, have reduced immunogenic risks. At the same time, *recurrent dosing* of such nanoparticles may elicit unique immune responses, e.g. potential immunogenicity of polyethylene glycol (PEG) that is often used as the tethering polymer ‘spacer’ for nanoparticle surface-decoration of peptides (137). Evaluation, optimization and mitigation of these aspects will require significant multi-disciplinary efforts between scientists, engineers, clinicians and regulatory experts, along with appropriate funding, to be able to ultimately utilize the immense potential of such technologies in saving lives.

Declarations of interest

ASG is an inventor on patents involving Synthetic Platelets (US 9107845, US 9636383, US 10426820, US 10434149). He is also a co-founder of Haima Therapeutics and chair of Haima’s Scientific Advisory Board (SAB). ASG is supported by the National Institutes of Health (NIH) R01 award numbers HL121212 and HL129179. The content expressed in this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. MDN is supported by NIH R01 award number R35 GM119526. MDN is a member of Haima’s SAB.

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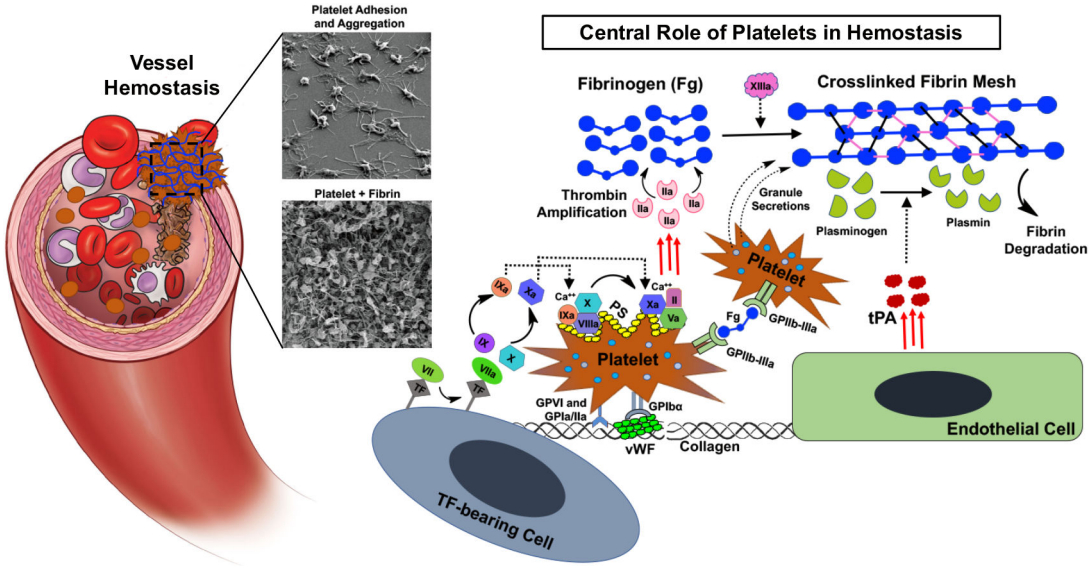

Figure 1.

Schematic showing the central hemostatic mechanisms driven by platelets involving adhesion to von Willebrand Factor (VWF) by platelet GPIb?, adhesion to collagen by platelet GPVI and GPIa/IIa (?2?1), aggregation via binding of fibrinogen to platelet integrin GPIIb-IIIa (?IIb?3), exposure of phosphatidylserine (PS) on activated platelet surface that renders the ‘thrombin burst’ for coagulation amplification and fibrin generation, and secretion of platelet granule contents to further enhance coagulation outputs; Scanning Electron Microscopy (SEM) images show characteristic adhesion and aggregation of activated platelets on a collagen surface, as well as formation of fibrin clot over activated platelets; The fibrin clot is ultimately degraded by the action of plasmin produced from plasminogen by the action of endothelial cell-derived tissue plasminogen activator (tPA).

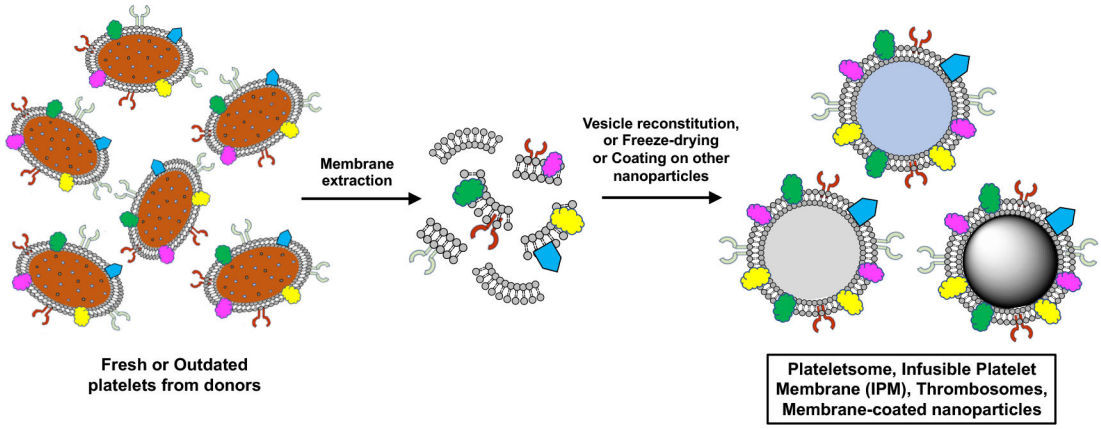

Figure 2.

Platelet surrogate designs derived from (and dependent on) natural platelets, especially via extraction and utilization of platelet-derived membranes that partially retain the functions of hemostatically relevant glycoproteins and lipids.

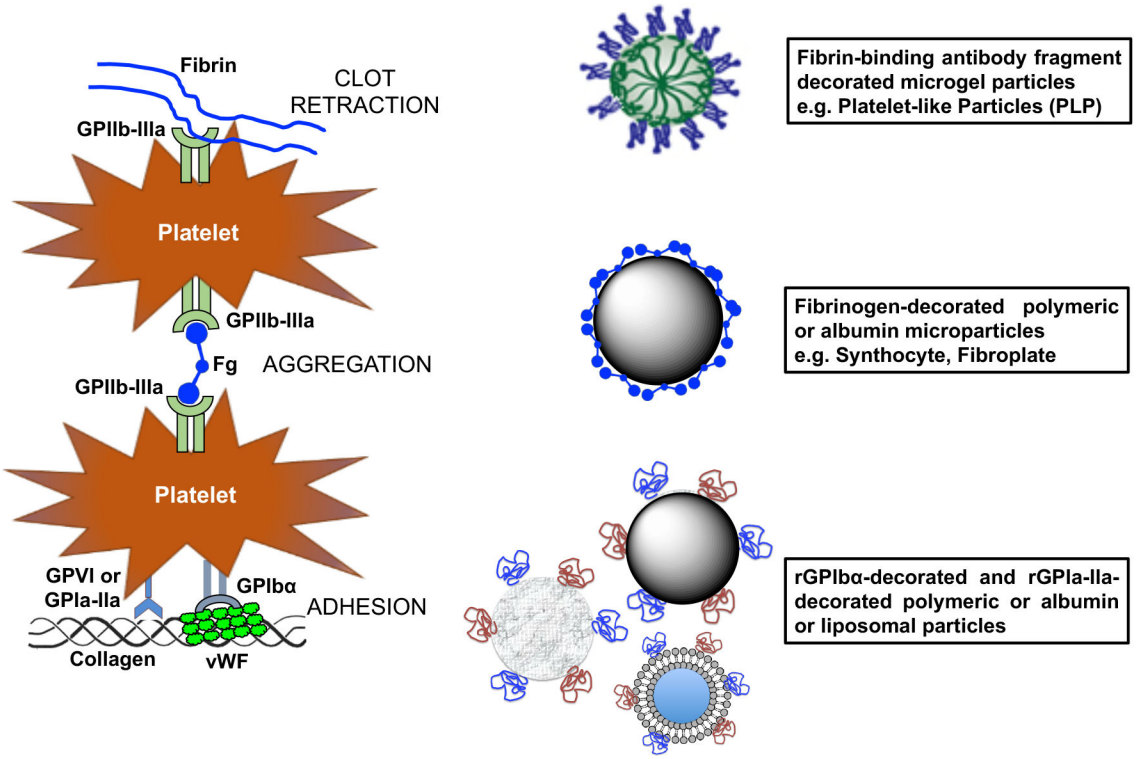

Figure 3.

Artificial platelet designs that utilize particle surface coating with proteins and antibody fragments to emulate various hemostatic mechanisms of platelets; Examples include particle surface-decoration with fibrin-binding antibody fragments or nanobodies to emulate platelet-fibrin interactions for clot retraction, particle surface-decoration with fibrinogen to enhance platelet aggregation, and particle surface-decoration with recombinant glycoproteins that emulate VWF- and collagen-adhesive mechanisms of platelets.

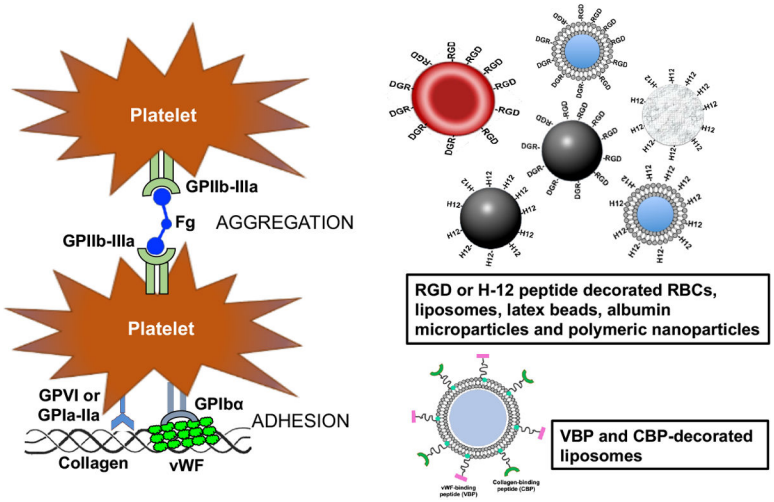

Figure 4.

Artificial platelet designs that utilize particle surface coating with peptides to emulate various hemostatic mechanisms of platelets; Examples include particle surface-decoration with fibrinogen-relevant RGD and H-12 peptides to augment platelet aggregation, and VWF-binding as well as collagen-binding peptides (VBP and CBP) to emulate platelet adhesion mechanisms.

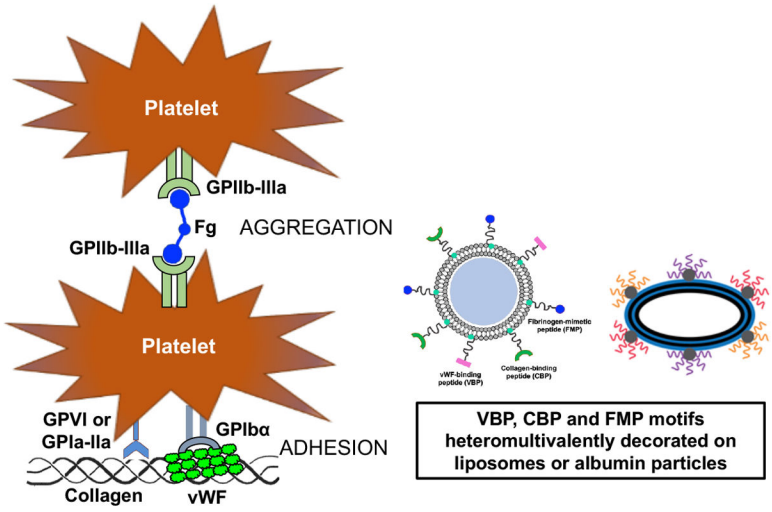

Figure 5.

Artificial platelet designs that utilize heteromultivalent surface decoration of particles to integrate the adhesive (VWF- and collagen-binding) and aggregatory (fibrinogen-to-GPIIb-IIIa binding) mechanisms of platelets in hemostasis; Examples include particle surface-decoration with VWFbinding, collagen-binding and fibrinogen-mimetic peptides (VBP, CBP and FMP).

Table 1.

Summary of 'Platelet Surrogate' approaches categorized by materials or design approach, representative *in vitro* and *in vivo* evaluation results, and representative clinical trial information (where applicable). Abbreviations: RT- Room temperature, TEG-Thromboelastography, ROTEM- Rotational Thromboelastometry, RCT – Randomized Controlled Trial, NHP – Non-human primate.

	Materials and/or Design Approach	***In Vitro* Preclinical Findings**	***In Vivo* Preclinical Findings**	**Representative Clinical Studies**
Cold-stored Platelets (CSP or CS-PLT)	Donor-derived platelets stored at 1°-6°C	• Platelet metabolism is slowed • Bacterial contamination risk is reduced • Increased clot strength, aggregation, and reduced soluble factor release assessed by TEG, aggregometry and ELISA. • Cold storage lesion: Increase in intracellular calcium, Shape changes, Clustering of GPIb on membrane, Changes in membrane glycosylation, Increase in P-selectin and phosphatidyl serine externalization	• CSP participation in clot formation and hemostatic function equivalent to RT platelets in both normovolemic and hypovolemic hemorrhagic models in rats • CSP were quickly removed from circulation by hepatic macrophages, in rabbit model. • Higher hemostatic function but reduced circulation time	NCT02495506 Effects of leukocyte reduced CSP in treatment of immediate postoperative blood loss in patients undergoing thoracic surgery in combination with extracorporeal circulation. NCT02754414 Phase I - Platelet survival and function after cold storage for 20 days comparable to 5 days at RT. NCT03787927 Phase I & II - Determine platelet function with dual antiplatelet therapy prior to transfusion of CSP vs RT platelets in healthy subjects NCT04834414 Phase III randomized storage duration ranging study in patients undergoing complex cardiac surgery comparing to RT platelets.
Cryopreserved Platelets (CPP)	Donor-derived platelets spiked with DMSO, frozen and stored at −65°C or −80°C, that can be thawed back for use	• Functional for 2-4 years • Reduced aggregation property but increased procoagulant activity by high exposure of phosphatidylserine and microparticle release • Reduced clot formation time in ROTEM studies	• In mouse traumatic hemorrhage model syngeneic CPP reduced clotting time and restored blood pressure • In thrombocytopenic dog model, canine CPP improved hemostasis	NCT03991481 Phase III multicenter blinded RCT non-inferiority trial of CPP vs. liquid-stored platelets in surgical bleeding NCT04709705 Randomized trial to evaluate the non-inferiority or superiority of CPP with liquid-stored platelets in cardiopulmonary bypass
Lyophilized Platelet Products (Lyo-PLT, Thrombosomes)	Apheresis-collected Group O 2-3 day old donor platelets trehalose-stabilized, lyophilized and stored at RT as powder that can be reconstituted in sterile water for on-demand use	• Thrombosomes maintain clot formation properties (ROTEM) and adhere to collagen under flow • Less microparticle content than other methods • Phosphatidylserine expression increased	• Thrombosomes exhibited safety and hemostatic function in rabbit ear bleed model • Reduced blood loss in canine CABG model. • Safety and efficacy in NHP hemorrhage model	NCT02223117 Thrombosome Phase I in normal subjects with dose escalation showed no serious adverse events. NCT04631211 Phase II multidose trial to evaluate Thrombsome in multi-dose in bleeding thrombocytopenic patients
Platelet membrane based 'Artificial	• Detergent-extracted platelet membrane incorporated on	• Plateletsome had no inhibitory effects on native platelet	• Plateletsome decreased tail bleeding in rat	• N/A for Plateletsome

	Materials and/or Design Approach	***In Vitro* Preclinical Findings**	***In Vivo* Preclinical Findings**	**Representative Clinical Studies**
Platelet' (Plateletsome, Infusible Platelet Membranes/IPM)	liposomes (Plateletsome) • Extracted platelet membrane, reconstituted as vesicles (IPM)	aggregation or coagulation outputs (PTT) • IPM increased platelet deposition and procoagulant activity in microfluidics based assays	model of radiation-induced thrombocytopenia • IPM decreased bleeding time in rabbit model of thrombocytopenia	• In normal humans IPM did not show adverse effects. Phase I and II trials showed decreased bleeding in thrombocytopenic human patients. No further studies or data available
'Artificial Platelet' designs with Adhesion Properties	Albumin, latex or liposome particle decoration with rGPIbα, or rGPIa/IIa, or CBP or VBP or their combinations	Effective particle adhesion on collagen-coated, vWF-coated or 'collagen + vWF'-coated microfluidic surface under shear flow	CBP and VBP-decorated particles reduced bleeding time in a mouse tail transection model.	N/A
'Artificial Platelet' designs with Aggregation Properties	RBC, albumin, latex or liposomal particle decoration with fibrinogen (Fg), generic RGD peptides (e.g. GRGDS), Fg gamma chain derived H-12 peptides, or Fg-mimetic cyclic RGD peptides (e.g. FMP)	• Fg-decorated RBCs (Thromboerythrocytes) showed ADP-induced aggregation and co-localization with platelets on collagen-coated microtiter wells • Fg-decorated albumin particles (Synthocytes, Thrombospheres) showed co-aggregatory capability with activated platelets • GRGDS-decorated particles increased platelet aggregation • H-12 peptide decorated particles enhanced thrombus formation on collagen • FMP-decorated particles enhanced platelet aggregation in well-plate and aggregometry assays	• Fg-decorated albumin microparticles reduced ear bleeding in thrombocytopenic rabbits, saphenous vein bleeding in thrombocytopenic mouse and improved survival in radiation-induced thrombocytopenia model in mouse • GRGDS-decorated polymeric nanoparticles reduced bleeding in rat femoral artery injury model and improved 1-hour survival in a mouse blast trauma model • H-12 peptide decorated particles shortened tail bleeding times in a busulfan-induced thrombocytopenia model in rats; Similar particles could deliver ADP for hemostatic effects	N/A
'Artificial Platelet' designs with hetero-multivalent integration of *Adhesion* and *Aggregation* properties (e.g. SynthoPlate)	Albumin or liposomal nanoparticles surface-decorated with combination of recombinant GPIbα and H-12 peptide, or combination of VBP, CBP and FMP peptides	• Heteromultivalently decorated nanoparticles showed enhanced adhesion to 'vWF + collagen' coated surfaces compared to homomultivalently decorated particles in microfluidic experiments. • Conjugating large protein fragments (e.g. rGPIbα) with small peptides (e.g. H-12 or FMP) for combination decoration of particle surfaces caused	• Heteromultivalently decorated nanoparticles bearing VBP, CBP and FMP (e.g. SynthoPlate) reduced tail-bleeding in antibody-induced thrombocytopenia model in mice • Heteromultivalently decorated nanoparticles bearing VBP, CBP and FMP (e.g. SynthoPlate) reduced tail-bleeding in antibody-induced	N/A

	Materials and/or Design Approach	*In Vitro* Preclinical Findings	*In Vivo* Preclinical Findings	Representative Clinical Studies
		mutual steric interference of their bioactive function • Combining small molecular weight peptides (VBP, CBP and FMP) in heteromultivalent decoration of nanoparticles (e.g. SynthoPlate) reduced steric interference of mutual bioactivity, enhanced platelet adhesion and aggregation in microfluidic assays and aggregometry when compared to homomultivalently decorated nanoparticles	thrombocytopenia model in mice • SynthoPlate reduced bleeding and improved 3 hour survival in a rat model of liver traumatic injury • SynthoPlate reduced bleeding and improved 1 hour survival in a pig model of femoral artery traumatic injury	