Bio-inspired manufacturing strategies for Platelet Analogues

Running title: Bio-inspired manufacturing strategies of Platelet Analogues

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Abstract

Blood transfusion is an important method in clinical treatment. The lack of blood donors and risk of contamination caused by blood transfusion has become a worldwide problem. Technology of mimicking platelets is imperative, with the greatest potential to significantly improve hemostatic action and break barriers of time and space. So that, many scientists have devoted to the research of artificial human hematopoietic cells. Imitations of the natural form and function of platelets are still limited by many reasons until now. In this review, we mainly focus on the constructive progress of platelet analogues based on its innate hemostatic abilities in the past 20 years. It hopes to convey a more comprehensive understanding of design elements, advanced technologies and major challenges in this domain.

Key words: platelet analogues; bio-inspired manufacture

1. Introduction

Natural platelets are used for emergency transfusions in urgently bleeding patients or to prevent life-threatening complications in patients with extremely low platelet counts. In the past few decades, a lot of research and development has been performed to assess the efficacy and safety of platelet transfusion. Even though platelet products have become of high quality, the therapeutic outcome of platelet transfusions is still largely affected by multiple immune and non-immune responses (*1*) . As a result, a vast extent of clinical data reveal that the desired therapeutic effect could not be achieved after platelet transfusions. In addition, there has always been a potential risk for pathogen transmission during the platelet transfusion process. To further protect patients from risks of unknown/emerging pathogens or of early infection in " window phase", some new technologies such as Mirasol pathogen reduction technology (Mirasol PRT), a chemical reaction between vitamin B2 and nucleic acids under the ultraviolet light, are more suitable for detecting viruses in transfusion-associated infections, but they also happen to reduce the efficacy of platelet transfusions, while increasing platelet consumption(*2*). In addition to the aforementioned drawbacks,

increasing clinical demands and human applied limitations (negative side effects, limited shelf-life and reduced donors) of natural platelet transfusions make it extremely urgent to design platelet substitutes for effective hemostasis (*3-5*). Current platelet derived products are associated with platelet surface receptors or their ligands (e.g., GPIb, GPIIb, VBP, vWF, and H12) and different kinds of platelet derived products were developed successfully, namely; lyophilized platelets, liposome-based platelet analogues, and polymeric nanoparticles(*3*, *6-13*). (See Table 1).

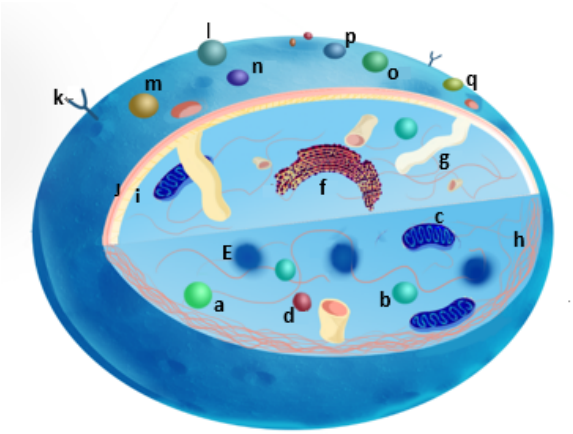
Previous reviews are mainly focused on biological functionalities, simple physical and mechanical characteristics. In this study, we narrowed the attention to bioengineering platelet analogues, and put forward a comprehensive discussion from various aspects such as structure, mechanical and physical properties, material selection, evaluation methods, animal models and current related research progress in order to provide valuable information for subsequent studies on artificial platelets. In order to design platelet analogues that are most physiologically similar to natural platelets, flexible physical performance synergistically combining favorable biological properties and evaluation strategies are discussed.

Table 1. Classification of platelet subsititues

Classification of platelet subsititues	Specific subsititutes
Platelet-based products (85)	● Platelet membrane microparticles ● Insoluble platelet membrane microparticles ● Frozen platelets ● Cold-stored(4°C)platelets ● Lyophilized platelets
Coated/Conjugated polymers (25, 26, 43-55, 57-65, 70-75, 77, 86-87)	● Albumin granule ● Latex bead ● Lipid vesicle ● Phospholipid vesicle ● Synthetic particle ● Liposome ● Microgel ● Nanoparticle/Nanosheet
Other analogues(66-68)	● polyphosphate nanoparticle (ployP NP)

2. Synthetic Design Parameters

Platelets originate from megakaryocytes, which are differentiated from pluripotent hematopoietic stem cells. On light microscopy, platelets appear as biconvex discoid (lens-shaped) structures, which promote their peripherization to the vessel walls. They have various glycoproteins and receptors on the surface (See Figure 1). When platelets reach damaged vessel walls, the glycoprotein GPIbα on their surface bind to the A1 domain of vWF on the exposed subendothelial matrix and platelets roll along the surface of the tissue. Simultaneously, the platelet surface glycoproteins GPIa-IIa and GPVI bind to the subendothelial collagen at the injury site, making platelet adhesion more stable. Platelets then bind to collagen and get activated by GPVI and agonists, such as adenosine diphosphate (ADP) and thrombin. When platelet adhesion-related receptors bind to their corresponding ligands, there is a series of changes of the activated platelets in terms of morphology, aggregation and particle release, which is important in hemostasis (*14*).



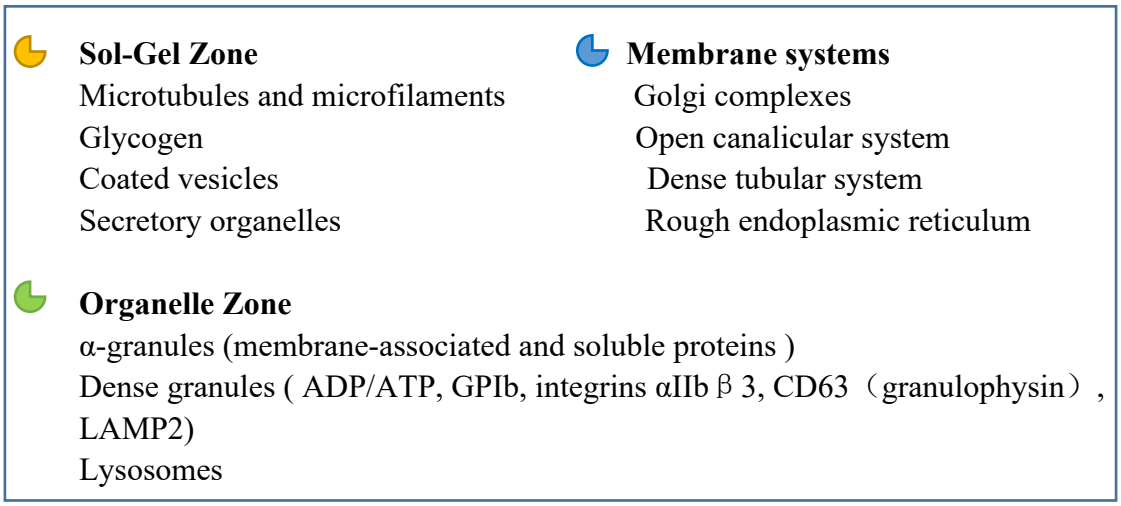


Figure 1. The structure of platelet is closely associated with its function. The platelet plasma membrane has a thicker glycocalyx with a wrinkled appearance that contains the surface glycoproteins (GPs) required for the interaction of platelets with subendothelial structures, platelet activation, platelet adhesion and aggregation, as well as clot retraction. The lipid bilayer and the submembrane region with a system of thin actin filaments—the membrane contractile cytoskeleton are below the glycocalyx. The former one contains tissue factor (TF), which is exposed to the platelet surface in an inactive form and plays an important role in enhance thrombin after the activation of platelets. The center of platelet is a granular zone with organelle aggregated and transparent zone surroundings, participating in various functions such as platelet synthesis and secretion.(a.lysosome b. glycosome c. mitochondria d. dense granule e. α-granule f. rough endoplasmic reticulum g. Open canalicular system h. microfilaments i. Actin filaments-the membrane contractile j. Lipid bilayer k. FcR l.GPIb-IX-X m. GPVI n.GPs o.αIIbβ3 p.CD3 q. Glut3)(83)

Platelets are activated to secrete their granular contents and accomplish their physiological roles (See Figure 2). Two adhesive proteins fibrinogen and vWF important for the process of platelet aggregation and adhesion respectively, and localized to the damaged vessel wall (*15*) . Fibrinogen is the ligand of the surface integrin GPIIb-IIIa which undergoes a conformational change and enables it to interact with any of the three peptide domains on fibrinogen (RGD motif: RGDF, RGDS; H12 sequence: HHLGGAKQAGDV) (*6*, *16*, *17*).

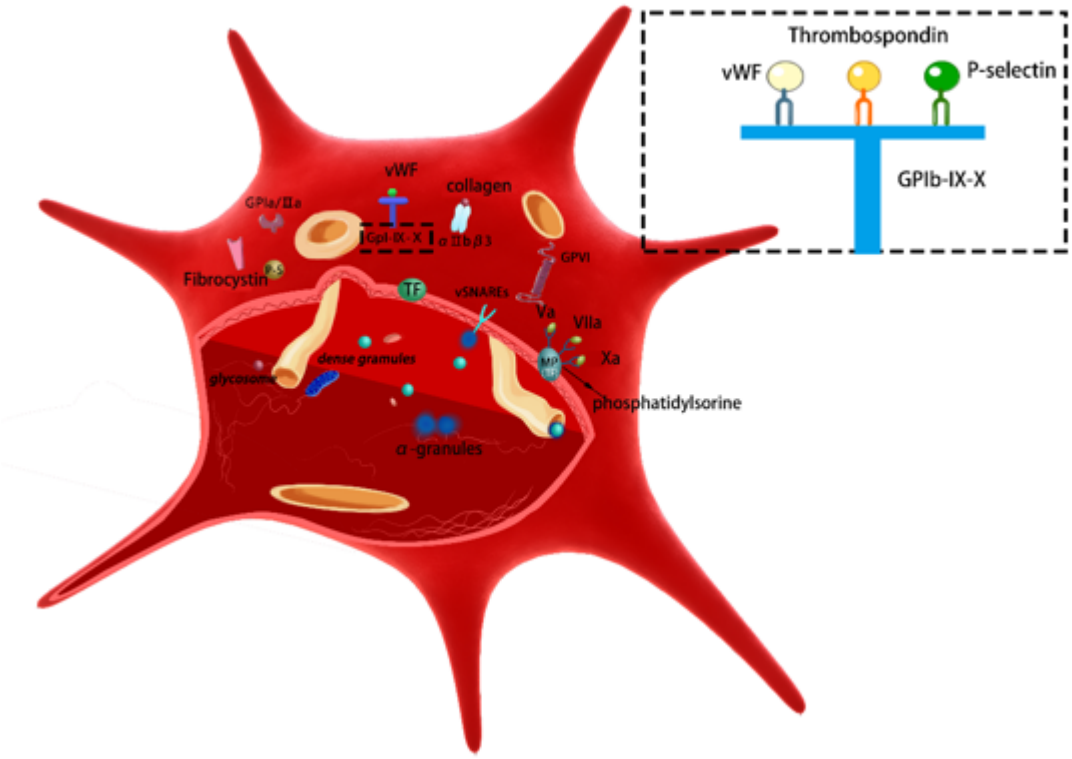


Figure 2. (A) When a platelet is activated, the appearance of it is changed from a shape of discoid to star. Channels of open canalicular system can be evaginated and enable to increase the surface area more than fourfold compared to resting platelets. Meanwhile, constricting microtubules which promote granules move to the center of platelet and release them to extracellular space through the channels. (B) GPIb-IX-X mainly achieves the adhesion of platelet-endothelial cells and platelet-platelet interactions through three different binding sites: vWF, thrombospondin and P-selectin.

2.1 Physical Characteristics

For platelets with ideal margination dynamics, strong marginal ability is critical to carry out physiologic processes in the bloodstream. Once the components (mainly collagen) from vascular endothelial cells are exposed to target receptors on platelet analogues, the adhesive propensity of these particles acts as a key determinant of therapeutic efficacy. The balance of interplay among ligand-receptor interactions between particle-endothelium and hydrodynamic shear stresses plays a vital role in particle adhesion, which can be modulated by the particles' morphology. Physical characteristics and biocompatibility of particles exert a crucial effect on their biological action, distribution in vivo and clearance (18) . Thus, the vascular environment should be taken into consideration when platelet analogues are designed because particles in

vivo are influenced by lots of complex factors (dynamic factors, active compounds).(19, 20).

2.1.1 Shape

Neutrally buoyant spherical particles require an external force to drift laterally. Whereas, non-spherical particles exhibit complex tumbling and rolling motions which replaces the need for additional external forces and these particles marginalized directly with higher efficiency(21, 22).

According to Decuzzi and Ferrari's defined adhesive strength parameter, the maximum adhesion volume of non-spherical particles significantly exceeds that of spherical nanoparticles in cases of comparable vascular shear stress, endothelial receptor expression and surface density of the targeting ligand. Some computational and mathematical models predicted that high aspect ratio carriers were easier to contact and firmly adhere to the luminal wall compared with volume-matched spherical particles under similar conditions (23) Therefore, Non-spherical particles have more advantages than spherical ones in mimicking the biological properties of platelets..

Nishit Doshi et al obtained similar results after comparing the particle adhesion of polystyrene spheres (3um) stretched into different shapes (elliptical disks, disks, spheres, flattened circular, and rods), and proved that the particles' shape determines their adhesion ability. Elliptical particles were more likely to show the adhesion than spherical ones, circular disks were in the middle, further indicating that elongation might be more important than flatness (24, 25) . Aaron C. Anselmo et al. demonstrated that discs and platelet-like particles adhered in higher quantities than spherical particles at the nanoscale. Platelet-like nanoparticles (PLNs) were more significantly displayed, probably owing to their flexibility and characteristic of adhering as both individual and aggregates instead of adhering only as individual particles like spheres and discs (26). Altogether, controlled geometries designed for function is a recognized delivery strategy. A disc-like shape similar to human platelets is associated with better

performance than a sphere regardless of material properties， especially the behavior of margination in flow (23, 27).

The shape change of natural platelets should be taken in to consideration as well (28) . As stated previously, platelets are transformed from a smooth discoid shape to a star shape when activated, with the latter displaying pseudopod protrusions and particle release. Platelet pseudopods increase the frequency of collision and minimize electrostatic repulsion between two platelets, thereby enhancing platelet interactions for aggregation (29) . Consequently, this star shape may be of great importance in the design of platelet analogues. Ashley C. Brown et al. created the deformable synthetic platelet-like particles (PLPs) in relation to their abilities of actively collapsing fibrin networks, which achieve wound-targeted hemostasis and decrease bleeding time in vivo with greater conformational flexibility (30).

Regarding platelets mimicking technologies, the stretching spherical particles (31) and mini-emulsion technique (32) may be two appropriate approaches among various methods for producing biomedical polymeric nanoparticles. The former one consists of suspending particles in an aqueous solution of polyvinyl alcohol (PVA) and casting them into films, then acquiring the final desired shape by manipulating the film accordingly. Moreover, the mini-emulsion produces different shapes in solution by the micelle self-assembly behavior. Shinji Takeoka et al. provided an approach to help develop platelet analogues, and they proved that rGPIbα-conjugated phospholipid vesicles showed the ability to roll on the vWf surface similar to that of natural platelets and indicated that increasing the membrane flexibilities of particles would lead to a decline in their rolling velocities. Regulating the membrane flexibility is a possible option to control the velocity of platelet analogues (33).

2.1.2 Size

In the design of physical properties, particles' size is the easiest element to control and implement. Due to filtration and biological distribution, the size of particles in different parts of body are limited to a narrow range and there is a loss of performance in blood circulation. Nanoparticles are widely used in the application of carriers due to

the enhanced targeting accuracy and reduced off-target side effects. As researchers considered clearance in the body and circulation time, the size of such particles are always limited from 2nm to 200nm (34). Randall Toy et al. explored the trend of particle marginalization at nanoscale. They compared margination of three different liposomes encapsulated with PBS (65nm, 100nm, 130nm) in a rectangular channel and demonstrated that smaller particles underwent higher deposition due to the inversely proportional relation between the diffusion coefficient and particle size (19). Aaron C. Anselmo et al. used the antigen-antibody model under flow conditions to compare spherical particles with different magnitudes (200 nm, 1 μm, and 2 μm). They discovered that the adhesion propensity of 200nm was the most significant due to the larger particles adhered in smaller quantities and that it may later suffer from the stronger shear detachment forces in the bloodstream through microfluidic devices (26). Yet in the study of Cooley et al, micron-scale particles were confirmed that they had certain advantages of margination compared to nanoscale particles under the influence of RBC. The volume fraction of RBCs and HCT is the most important factors in particle margination. RBC will fill more space in vessel center and shove other components towards to the wall (34) . Altering the diameter of differently shaped particles would cause them to have different adhesion trends in the device. Nishit Doshi et al. tested the adhesion propensity of spherical particles with three different sizes (1μm, 3 μm and 6 μm), and the 1μm particles exhibited the most significant ability of adhering to the vessel wall through synthetic microvascular networks. On the other hand, the adhesion of non-spherical particles rose with an increase in the diameter of particle (24), Phapanin Charoenphol et al. found that the sphere's binding efficiency to the endothelial wall from 0.5nm to 10μm increases with augmentations in the size, wall shear rate and channel height through the parallel plate flow chamber. Particles with different sizes have different performances in different size channels and under different shear rates. The adhesion of larger particles occurred in reaction-limited region (RLR) at higher wall shear rates, which resulted in reduced binding. Micro-sized spheres displayed lower adhesion propensities in models imitating small and medium-sized blood vessels and spheres with 2–5μm size are optimal carriers for medium to

large vessels especially in cardiovascular diseases (35, 36). Overall, it appears that the appropriate size of platelet analogues is not limited to one specific size. Instead, multiple specifications for platelet substitutes may be a trend which depends on a set of factors, namely; ability of marginalization, adhesion, received forces of particle motion and biological distribution for adapting to various needs.

2.1.3 Density

The shape and density of nanoscale particles used in biofacturing platelets could be engineered to adjust the marginalization propensity. Randall Toy et al. examined the effect of particle momentum by comparing different spherical particles (liposome, iron and gold) of the same size (65nm) and different masses, indicating that the momentum of nanoparticles with the same size was strongly dependent on the particles' density. When compared with low density models, higher density nanoparticles carried more momentum and harder to marginalize(19). Gentile et al demonstrated the opposite result, stipulating that large density particles (16um, quasi-hemispherical silicon particles) displayed better tendency to marginalize than the small density ones at a given shear rate (37). It may be because of the difference in materials, diameters and devices.

2.2 Factors of dynamic environment

The extrinsic factors influencing particles (flow rate relative density, affecting buoyancy, Hamaker constant A) in a dynamic vascular environment are the crucial parameters, which equally influence the marginalized ability of particles. Randall Toy et al. tested and ascertained that a 65 nm liposome would be deposited more easily at a slower flow rate (50 μL/min) than at a faster one (200 μL/min) , because the fibronectin lacked sufficient adhesive strength to maintain these deposited particles onto the wall of the model at a faster flow rate(19, 38). Decuzzi et al. analyzed four types of forces experienced by particles during blood flow, (hemodynamic forces, buoyancy force, van der Waals interactions, electrostatic double layer (EDL) interactions and steric repulsive interaction), then summarized a function of the geometrical and material properties of the system which can be used to estimate the time of marginalized velocity

of particle and contacting the endothelial wall. Research showed that the diameter of a delivered particle into the bloodstream needed to be limited lower than the critical value to facilitate margination and interaction with the endothelium (39). (Figure 3) AlMomani et al. studied the interaction between platelets and RBCs in the dynamic flow environment by using computational fluid dynamics (CFD) models. An extension of the soft sphere model for elliptical particles was used to simulate the forces and torques between the colliding blood cells. They finally highlighted that migration of platelets was directly proportional with increase in the cell hematocrit (40). Most platelet fluid mechanics studies were limited to a single platelet shape. However, Dupin et al. designed a multi-component lattice Boltzmann method to acquire an efficient computational technology for simulating a tremendous quantity of deformable particles, which provided a new potential model of platelet mimicking applications (41).

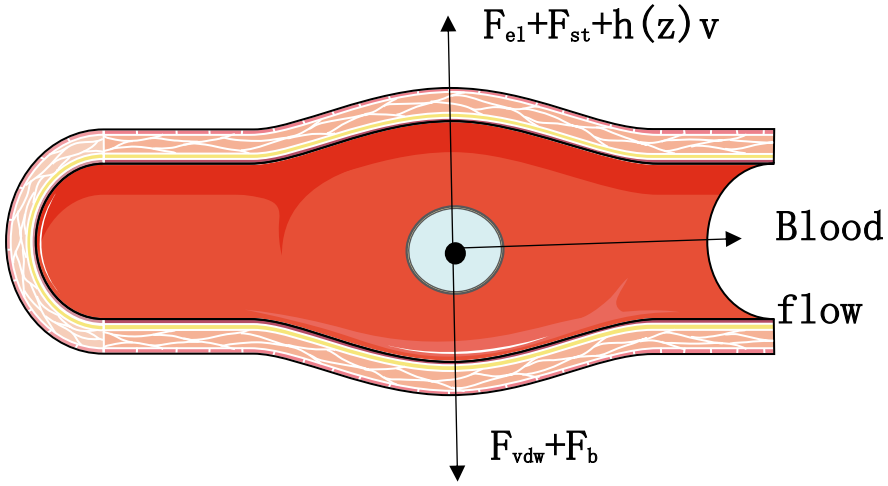


Figure 3. Applied forces of a particle in blood flow, (Fel force due to the electrostatic double layer interaction, Fsr force due to steric repulsion, FvdW force due to the van der Waals interaction, h due to hemodynamic resistant function, v due to steady velocity, z due to z-axis coordinate for the center of the particle)(39)

3. Bioengineering Strategies for Platelet Substitutes

The research on artificial cells has existed for 60 years now and Chang et al. have achieved satisfactory results in artificial red cells. Combining the early research on artificial human cells and the shortage of natural platelets, the researchers have focused on the hemostatic process of platelets and came up with a lot of further design ways of platelet analogues. The followings are some important time nodes which provide the

necessary theoretical supports or significant achievements on platelet analogues (*42*). (Figure 4)

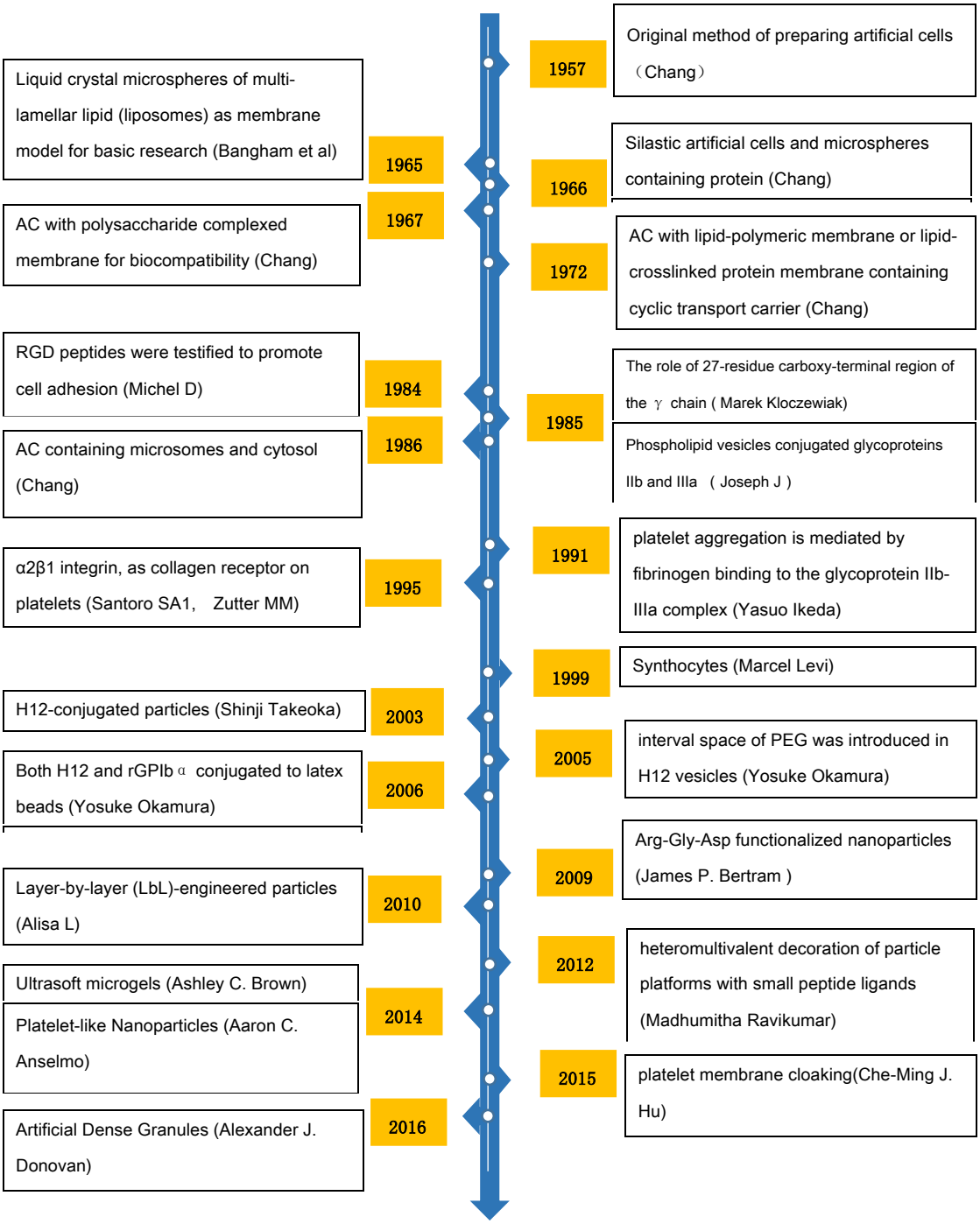


Figure 4. Timeline for providing the necessary theoretical support or significant results for artificial platelets (26, 42, 43, 45, 47, 48, 52-54, 62, 63, 69, 70, 73, 77, 88-91)

3.1 Fibrinogen-based Microparticles

Fibrinogen is converted to fibrin through thrombin enzymatic reaction during the damage of. tissue and blood vessel which plays a fundamental role in the coagulation process. Levi et al. developed an artificial microcapsule coated by fibrinogen (Synthocytes) as platelet substitute products, which could promote adhesion of new particles and prolong the bleeding time in rabbits (*43*). These approaches were limited so as to concentrate and imitate either the function of platelet adhesion or aggregation, but not both (*44*).

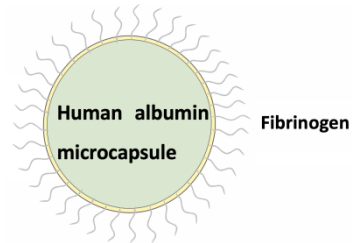
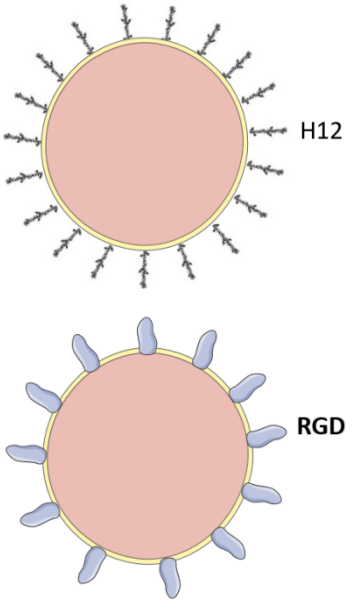
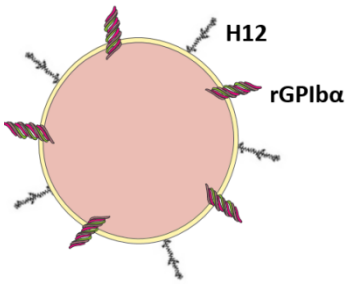
3.2 H12/RGD-coated Platelet Substitutes

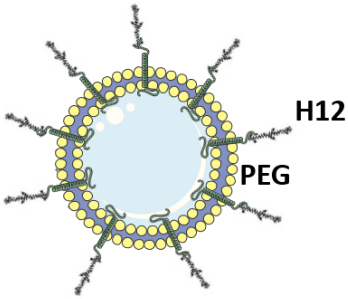
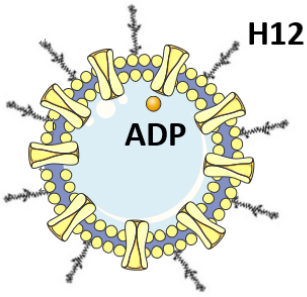
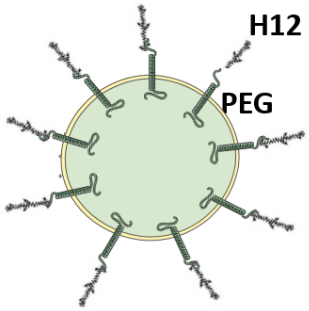
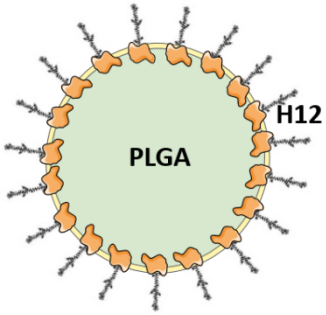
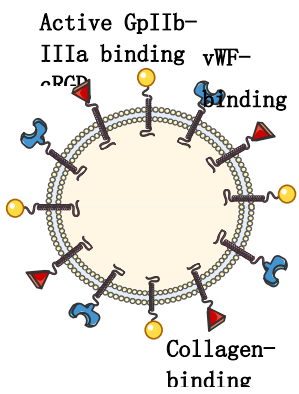
Fibrinogen is a glycoprotein in human blood circulation and essential for platelet aggregation. It is composed of three pairs of polypeptide chains (α, β, γ) maintained together in a covalent structure(45) and three platelet interaction sites are at the chains. Dodecapeptide (HHLGGAKQAGDV, H12) is the sequence at γ-chain carboxy-terminal segment and a tetrapeptide containing RGD sequences are at α chain. The sites recognize the active form of glycoprotein IIb/IIIa on membranes of activated platelets and performance better in enhancing the biocompatibility and stability of synthetic vesicles than fibrinogen-coated ones(46, 47).

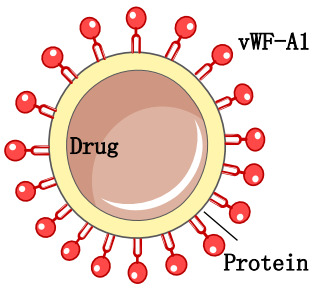
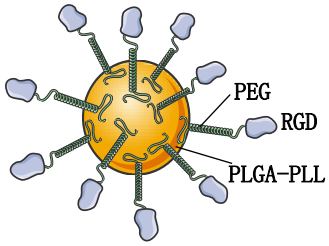
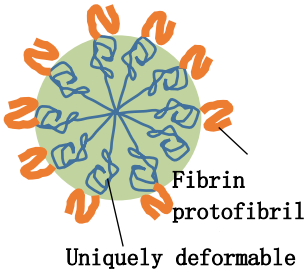
There are various studies of synthetic platelet analogues in which the fibrinogen-derived H12 and RGD peptides were focused. (Table 2) Also, particles modified with the combination of platelet aggregation-promoting H12 and vWF adhesion-promoting recombinant GPIba motifs were found to exert significantly higher hemostatic functions, compared with separate modifications of either one(44). Shinji Takeoka et al. prepared particles with two oligopeptides (H12 or RGD) and conjugated them to latex beads with a recognizable site (an added amino-terminal cysteine) which targeted activated platelets for thrombus formation. Compared with RGD conjugated latex beads, H12 conterparts showed a minimal interaction with non-stimulated platelets, even under the suppression of PAC-1 and the anti P-selectin antibody(47). Researchers paid more attention to H12 and tried different combinations in order to achieve a greater breakthrough. Yosuke Okamura et al. have done in-depth research in this field, mainly focusing on four kinds of carriers (latex bead, phospholipid vesicle, polymerized

albumin particle, nanosheets). As far as latex is concerned, something different from the above research is the addition of rGPIbα, which adhered on the surface of collagen at a wide range of shear rates and displayed a synergistic effect at a high shear rate, while the function of H12 and rGPIbα in conjugated latex beads were limited by steric hindrance (48).

Table 2. The products of H12/RGD-coated platelet substitutes

Name	Diameter	Important factors of design	Simple structure	Reference
Synthocytes	3.5-4.5um	human albumin microcapsule coated with fibrinogen		(43)
H12- or RGD-conjugated latex beads	200nm or 1 μm	Latex beads coated with human serum albumin （rHSA） and conjugated with H12 or RGD		(47)
H12/rGPIbα-latex beads		Latex beads coated with human serum albumin （rHSA） and conjugated with H12 and rGPIbα		(48)

H12-PEG-vesicles	220 nm	phospholipid vesicle conjugated with H12 and PEG		(52)
H12-(14C-ADP)-(3Hvesicles	-	Phospholipid vesicle conjugated with H12 and then ADP was encapsulated in		(51)
H12-polyAlb，H12-PEG-polyAlb	260±60 nm, 200 -80 nm，	polymerized albumin particles conjugated with H12 or H12/PEG		(50, 53)
H12-PLGA microparticles	2.2 ± 0.5 μm	Nanosheets prepared by biodegradable poly(D,L-lactide-co-glycolide) (PLGA) and H12 was conjugated on the surface		(77)
liposome model	150nm	conjugated with von Willebrand Factor (VWF)-binding peptide (VBP), a collagen binding peptide (CBP) and an active platelet GPIIb-IIIa-		(59)

		binding cyclic RGD-based fibrinogen-mimetic peptide (FMP)		
synthetic particle (SP)	1-1.5μm	coated with VWF-A1 targeting drug delivery to platelets		(25)
PLGA-PLL-PEG	1500RGD (SEM 178 ±68nm,DLS 326±45nm); 4600RGD (SEM 154 ±36nm,DLS 345±81nm)	PLGA-PLL core with PEG arms terminated with the RGD moiety		(63)
PLP	~1 μm	H6 sdFvs were conjugated to ULC μgels		(70)

They also introduced phospholipid vesicles as carriers and conjugated H12 to the end of the synthesized PEG lipid derivatives, which were connected by elected L-Glutamic acid, resulting in the formation of H12-PEG-vesicles. Due to the modification of PEG, the half-life of H12-PEG vesicles was significantly prolonged. Additionally, high-concentration PEG-vesicles could seriously reduce the bleeding time in thrombocytopenic rats models (49). Another one was H12-(ADP)-vesicle with different lamellarities and membrane flexibilities, which could augment platelet aggregation essentially by releasing ADP. It is definitely worth mentioning that the lamellarities

were controlled by main lipid component extrusions and the hemostatic function was controlled by membrane deformability. Radioisotope labeling method was used to showed that H12-(ADP)-vesicles release ADP in an aggregation-dependent manner to enhance platelet aggregation, and demonstrated successful hemeostatic ability by releasing ADP in a busulphan-induced thrombocytopenic rat model(50, 51). Similar performances were also observed in other combinations such as H12-polyAlb, H12-PEG-polyAlb and H12-conjugated disk-shaped nanosheets (H12-PLGA (biodegradable poly) (D,L-lactide-co-glycolide) (49, 52, 53)

3.3 Liposome-based Platelet Substitutes

In 1985, Joseph et al. documented their successful reconstitution of platelet glycoproteins IIb and IIIa into lipid vesicles and functional properties of these vesicles similar to those of intact platelets and isolated platelet membranes(54). Later on, some researchers continued to emphasize their investigations on liposomes based on the interaction of rGPIa/IIa–collagen and rGPIbα-vWF (55-57). Coupling VWF-A1/GPIbαN with respect to the simulation of platelet structure can significantly enhance the thrombus targeting with microparticles(27). Due to the effectiveness and superiority of platelet-mimetic design with both adhesive and aggregated properties, some studies are dedicated to overcoming the remarkable challenges of simultaneously binding various large protein fragments to one particle in spatial interference (44, 58)

Christa L. Modery et al. obtained a positive outcome of platelet mimetic design with highly selective to the injury surface of blood vessel. They produced a liposome coated with an active αIIbβ3 targeting the RGD peptide and P-selectin targeting peptide DAEWVDVS on its surface. This construct could promote adhesion and aggregation of activate platelets (58). Two years later, they produced a liposome model (diameter of 150nm) decorated by heteromultivalent small peptide in order to avoid spatial interference, this model was conjugated with von Willebrand Factor (VWF)-binding peptide (VBP), a collagen binding peptide (CBP) and an active platelet GPIIb-IIIa-binding cyclic RGD-based fibrinogen-mimetic peptide (FMP). Hence, VBP and CBP promoted the adhesion and FMP enhanced the aggregation of liposomes at the vascular

injury sites, which depicted obvious advantages in reducing the bleeding time and promoting hemostasis in vivo, compared with either promoting just adhesion or aggregation(59). Madhumitha Ravikumar et al also published the similar liposome-based design (60, 61).

3.4 Polymeric Microparticles

Nishit Doshi et al. designed a completely new synthetic particle (SP) to simulate natural platelet, whose physical properties were controlled through lithographic techniques and the film stretching method. They reproduced the ability of specific and distinct adhesion by exploiting the GPIbα/VWF-A1 bond for identifying platelet and relevant platelet substrate. Meanwhile, SPs were proved to have some success in treating bleeding by thrombus formation as well as increasing the thrombus volume of endogenous platelets and eventually improving the efficiency of hemostasis (25). The design of Hassan Haji-Valizadeh et al. had a different binding site of FVIII-binding vWF's D'-D3 domain and FVIII-derived VBP (residues 2303–2332), which could not interfere with the binding of natural platelets and was thrombin-cleavable (62).

James P. Bertram et al. engineered a synthetic platelet (PLGA-PLL-PEG-RGD) comprised of a poly(lactic-co-glycolic acid)-poly-L-lysine (PLGA-PLL) core with polyethylene glycol (PEG) arms terminated with RGD functionalities, this synthetic platelet only bound to the activated platelets to avoid adverse complication of nonspecific binding or induced platelet activation and effectively cleared within 24 hours at a dose of 20 mg/ml(63). Andrew J. Shoffstall et al. also developed intravenous effective hemostatic nanoparticles (GRGDS-NPs), while the risk of particle aggregation in lungs needed to be further assessed(64). Aaron C. Anselmo et al. made a significant improvement on bioengineering the artificial platelet-like nanoparticles (PLNs) which possessed a flexible capsule mainly due to the flexibility of the polymer/protein shell and layer-by-layer (LbL) approach. PLNs were decorated with CBP, VBP and the FMP and showed superiorities of marginalization, targeting adhesion and promoting hemostatic aggregation whilst augmenting adhesion to locating vascular injury site. LbL-engineered particles combined with different

polymeric systems and showed a huge potential in designing platelet substitutes (26, 65). The capacity to decrease the bleeding time was evaluated in a mouse tail transection model (26). Alexander J. Donovan et al. (66, 67). introduced a granular polyphosphate nanoparticle (polyP NP) as the core in the biomimetic nanoparticle. There was a remarkable resemblance between this core and natural platelet dense granules. Besides, synthetic core triggered the FXI to autoactivate, which had further possibility and broad prospects of engineering artificial platelets(66, 67). Further studies conjugated it to colloidal gold nanoparticles (GNPs) and potentially targeted to bleeding sites as a procoagulant agent (68).

3.5 Fibrin-binding Microgel Particles

Ultralow crosslinked (ULC) particles of microgels are yet another set of unique material introduced in the study of platelet analogues that have been used in drug administration. These particles are colloidally stabilized hydrogel microparticles with adjustable mechanical and chemical properties. They are characterized by their immense degree of deformability, have their sizes adjusted and charge by changing the reaction conditions as well as be synthesized without any necessity for a crosslinker. On the other hand, their degree of deformability must be kept under control in order to prevent unforeseen circumstances (69). An entirely synthetic platelet-like particle (PLP) was created by combining highly deformable gel particles and molecular recognition motifs based on introduction of material principally through overcoming the difficulty of adapting to deformation in the fibrin network, and it was demonstrated that the novel particle played a role in the process of clot contraction, achieved the function of platelets simulation that targeted the wound and effectively reduced the bleeding time of wound injury model. In addition, PLP exhibited an emerging behavior of simulating clot contraction and collapse an active fibrin network, which was linked to the deformability and affinity of fibrin fibers (70).

3.6 Cell Membrane Coating Particles

With the widespread use of bioengineering technologies in platelets simulation, it is strongly believed that an effective biological interface is a prerequisite for successful

in vivo translation. Biomimicry follows the naturally occurring strategies and is a synthetic nanoparticle coated with natural cell membrane by a facile top-down method(71-74).When cell membrane coating technology was first introduced in 2011, its subtle design and long-term natural circulation in mouse models began to take notice(73) and red blood cell membranes were the initial attempts(RBC-NPs) with different cores(75). Based on the previous successful results, platelets have also been studied as unique sources of cell membrane. Platelet membranes are obtained by repeated freeze-thaw cycles and coated on a polymeric PLGA core to form platelet membrane-coated nanoparticles (PNPs) which carried complete platelet surface markers while maintaining the initial functions of platelets without any appreciable toxicity. Besides, PNPs enhanced therapeutic effect in the atherosclerosis rat model and mouse model of systemic infection because they selectively adhered to blood vessels and enhanced to binding to platelet-adhering pathogens respectively (74).

4. Evaluation of Artificial Platelets

The FDA discussed the criteria for the approval of platelet products at the Blood Products Advisory Committee (BPAC) in July 2004.Most members suggested that the platelet analogues might exert a therapeutic effect on the human body rather than the salvage (recovery rate ≥ 66%, survival rate ≥ 66% is not clear), compared with fresh autologous platelets (76). Many techniques are available to evaluate the properties of platelet substitutes as mentioned below:

4.1 Perfusion chamber

Several models are designed for investigating carrier characteristics and simulate true vasculature environmental conditions. Synthetic particles are injected into the perfusion chamber with the help of a pumping device and every image of particle deposition can be monitored realtime through a microscope. Moreover, nanoparticles can be collected and calculated by measuring the volume. For instnce, the PDMS microchannel device (19, 24), microfluidic devices, which use the principle of antigen-antibody binding system or inner wall is endothelialized to highly simulate the

hemodynamic environment (26, 70). The recirculating chamber plays a pivotal role in observing the platelet analogues, whilst collagen and epifluorescence microscopy can be used when necessary (33, 52, 77), A parallel plate flow chamber (PPFC) with a straight channel(35, 36), was designed based on the simple flow chamber (37, 38), which was the most widely used device. Nonetheless, more adhesive events were noticed to occur in the chamber than in the microvasculature environment due to their comparatively larger size. Despite the fact that the the microchannel is desirable for its similarity in size, it is also limited by convolutions and bifurcations. Bifurcating synthetic microvascular networks (SMNs) may overcome both of these difficulties (24). Clarifying the exact role of the shape requires a comprehensive analysis of the experiments of more systematic designs and needs to integrate the shape parameters with in vivo functional properties (78).

4.2 Flow cytometry

Binding to monoclonal antibodies or other markers allows a precise study of the existed structure on the surface of platelet membranes by flow cytometry (79). Furthermore, adhesion can be quantified and screened out, meanwhile a targeted aggregation of platelet particles can be observed as well (51, 58).

4.3 Others

In addition to the aforementioned methods, there are also several approaches which can be used to evaluate the adhesion and aggregation of platelet analogues. Scanning electron microscope is used to observe the adhesion process directly owing to the high resolution of emerging microscopy techniques (46, 48). Theoretically, micro-scale model may be the most appropriate approach to evaluate the interaction between cells (40). In case exploring the distribution of biological particles in the body is necessary, fluorescence would undoubtedly be a more reliable and safer approach than radio nuclide assay (63). As for aggregation, aggregometers have been extensively used to measure the light transmittance of adding an ADP or collagen solution to a mixed platelet-rich plasma (PRP) (47, 48, 50, 52).

5. Animal Models

Due to the heterogeneity and complexity of patients with clinical trauma, it is necessary to evaluate the efficacy of platelets using experimental studies of various animal wound- healing models. Most of these test animals had artificially established wounds, followed by infusion of platelet analogues to observe variations in the bleeding time. Mice match approximately 80% of the human genome, and its specific advantages result in a common use in experimental wound studies. Nevertheless, compared to mice, wound models of rats and rabbits are technically easier to perform. In addition, because of their genomic distance from humans, further studies on large animals are needed before the results of experimental studies can be transferred in clinical trials. The similarities and accessibilities of hemodynamic responses and wound healing between pigs and humans make them widely used in the evaluation of wound-healing models of platelet analogues. We have listed animal models and basic information for studying platelets potency here. (Table 3) Conducting comparative studies on different animal models would also prove to be beneficial. That said, it must be realized that there is some potential limitations when trying to shift the results of the experimental models to clinical applications.

6. Clinical Aspects

It was found that patients with clinical thrombocytopenia rarely bleed, and relevant studies have shown that these patients have a significantly lower risk of hemorrhage with platelet counts above 2000/ml, and more clinical research have delineated that patients with acute myeloid leukemia are relatively safe even with platelet counts below 10,000/ml(80). Charles A. Schiffer further suggested that the increase in platelet count after transfusion is generally considered a measure of platelet viability. However, the platelet count after transfusion does not increase for some non-cellular platelet substitutes. It is imperative that its efficacy be determined by using a unique method. IIt is essential to prove the viability and effectiveness of artificial platelets in the human body (80-82).

Evaluating the hemostatic function of platelet substitutes is an important final purpose and the most universally used parameter to accomplish that is the period of time required to stanch bleeding from a standard skin incision. When the platelet count plummets to less than 10,000/μL, the bleeding time becomes unmeasurable after exceeding 30 minutes(81). Notwithstanding, the method of assessing efficacy of platelet transfusion using skin bleeding time is still being questioned. It is well known that the hemostatic function of platelets partly depends on the platelet count, size and biomass. A study on patients with familial hereditary thrombocytopenia and thrombocytopenic purpura showed that their bleeding time remains within normal limits even there was a significant reduction of platelet count, meanwhile bleeding time was prolonged in patients with leukemia or thrombocytopenia. Platelet efficacy may be a function of platelet kinetics, with younger platelets being able to normalize the bleeding time in lower numbers compared to older platelets. In addition, no clinical trial have proven that bleeding time can predict the risk of spontaneous bleeding (based on prevalence) and evaluate the effect of transfusion therapy(8). Hence, exclusively relying on bleeding time to evaluate the hemostatic effect of platelet analogues in the body could be both misleading and erroneous.

Table 3. Animal models for platelet analogues

Experimental subject	Advantages	Disadvantage	Basic condition	Injured part	Main measurement index	Reference
Porcine	1.highly sensitive to nanoparticles;2.most resemble to humans(hemodynamic, respiratory, skin);3.sensitive to complement activation and related pseudoallergy (CARPA);4.morphological similarity to human	1.pulmonary hypertension in response;2. low specificity to some liposomes	15-40kg	not mentioned	C activation-related pseudoallergy(CARPA) test (cardiopulmonary, hemodynamic, skin, hematological and blood	92,93
			≤26kg	muscle crush injury	hemorrhage volume	94
			30-40kg	vena caval injury	hemorrhage volume	95
			28-35kg	hepatic injury bolt gun	intra-abdominal hemorrhage volume(main index)	96
			45-55kg,3 to 4 months	spleen injury by wires	intra-abdominal hemorrhage volume	97
			Female	left midshaft femur	blood loss	98
				rectus abdominus muscle soft tissue crush injury	blood loss	99
			≤25kg	liver injury	blood loss	100
			40–50 kg	liver and spleen injury	blood loss	101
			not mentioned	femoral artery injury	blood loss, hematological index, biodistribution analysis	102
Nonhuman primates	similar to human body	1.complex anaesthetic process;2.lack of animal source	macaque	liver injury	blood loss	103
			baboons,23-33kg,AM	resection of the proximal clavicle plus a laparotomy	the biologic activity of thoracic duct lymph	104
Rats	1. small size;2. low cost;3. ease of handling;4. ethical acceptance;5. availability;6. easier operation than mice;7. share 90% of genome with human		250-280g	51Cr-activity of labeled platelets was injected into the tail vein	Platelet count, fibrinogen, fibrin monomer, and plasma hemoglobin	105,106
			230-250g,M	tail injury	bleeding time(tail)	107
			400-475g	tail injury	bleeding time(tail);blood loss(tail)	108
			400-475g	liver injury	blood loss	109
			200-250g,F	tail injury	bleeding time(tail);blood loss(tail)	110
			200-250g,M	tail injury	bleeding time(tail)	111
			300g, M	tail injury	bleeding time(tail)	63
			not mentioned	major femoral artery injury	bleeding time	43
Rabbits	1.decreases the variance and permits greater reproducibility of results	1.the vessel damage sustained during the isolation of the jugular veins;2.small vessels were disrupted	not mentioned	ear injury	blood loss	112
			2.5kg, 11 weeks old,F/M	ear injury	bleeding time(ear),PT, APTT and Fbg	113
			2.5-3kg	ear injury	bleedig time(ear)	114
			2.5-3kg;equal F/M,Thr(irradiation)	jugular veins	bleeding time	115
			2.5-3.5kg;AM;Thr(irradiation, heterologous platelet antiserum infusion, or a combination of both.)	Jugular Vein, Microvascular	bleeding time	43
			approximately 2.5 kg,Thr(busulfan-induced)	ear injury	bleeding time,blood loss(ear)	116
			2.5-3.5 kg,Thr(combination of irradiation and heterologous platelet antiserum infusion)	ear injury	bleeding time	118
Mouse	1.small size;2.low cost;3.easily handing;4.ethical acceptance	1.lack of standardized model;2.unstable;3.low matching rate of gene(80%);4.difficult operation;5.limitations of perianaesthetic management	not mentioned	tail veins	1.blood loss;2.hemoglobin concentration;3.the ability to survive the tail bleed	106,109
			C57BL/6 WT mice (8-12 weeks, male)	lacerted liver injury	blood loss, bleeding time	117

James N. George analyzed the benefits and risks of platelet substitutes or platelet components and posed that transfusion of platelet analogues might interfere with the function of autologous platelets and increase the risk of bleeding, and there is a lack of relevant clinical data. Moreover, the long-term risk of thrombosis in human beings is unpredictable. The use of any types of platelet components or substitutes, regardless of its efficacy, would prove successful if it prevents every episode with a maximum of three episodes within 15 years (83), therefore controlling the risk of platelet substitutes may be a more fastidious and long-term work than the study of synthetic particle structures.

8. Conclusion

In addition to playing a pivotal role in blood clotting and hemostasis, platelets also perform multiple physiologic tasks, including regulation of immune function through interaction with pathogens and cytokines, release of growth factors, immune concealment, and regulation of cancer metastasis (28). Due to the aforementioned issues associated with the clinical application of natural platelets, studies on platelets analogues have profound clinical implications. In the past 20 years, a variety of research designs on artificial platelets have been put forward, some of which have also applied for patents, which provide the basis and references for theories and methods. These research designs can be classified into two categories based on their respective directions. One of them is achieving large-scale production of mature blood cells derived from hematopoietic stem cells by inducing differentiation into platelets in vitro culture of megakaryocytes (MKs). The other is simulating the physiological functions of platelets to synthesize artificial particles via bioengineering techniques. (Table 4) Figure 5 is suggested design factors of platelet analogues. At present, platelets bio-manufacturing is excellent in the in vitro transformation of megakaryocytes, with a long-term clinical potential expectancy, providing sufficient resources to overcome platelet deficiency for clinical transfusion (84). While the current research on platelet analogues meet the bottleneck, artificial constructs cannot undergo morphological changes when activated like natural platelets and showed obvious weakness for

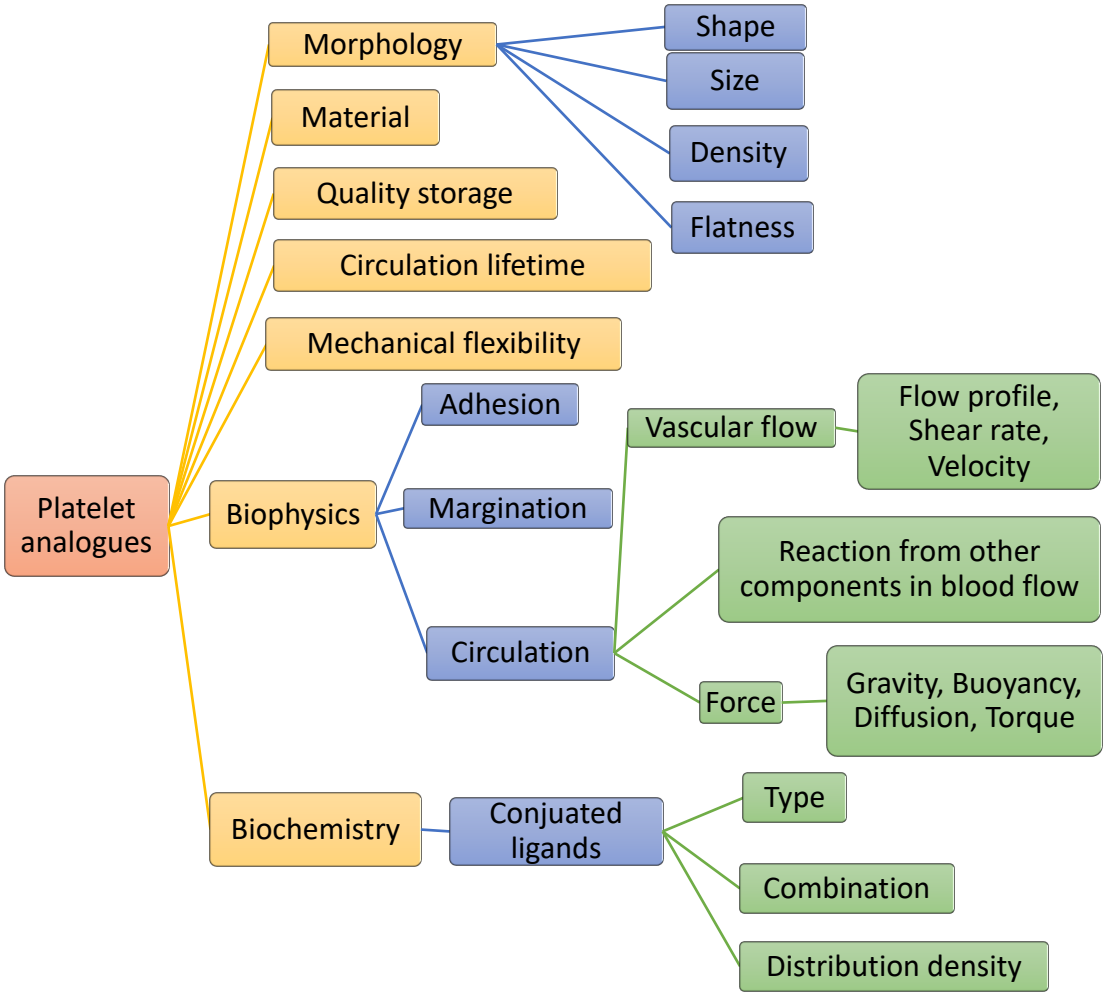
adapting to dynamic vascular environments (31). The design of "shape adaptive" platelet analogues is definitely an enormous challenge faced by the simulating platelet technology that has provided more ideas and technical support for drug-loading projects.

Table 4. Current Research Patents of Platelet production

Title	Inventor	Patent Application	filing date	Country
platelet-like proteo-microparticles and method of using such in drug delivery	Hsieh; Patrick C.H; Cheng; Bill	20180092846-A1	20 Apr 2016	Taipei
CROSS-LINKED PLATELET MATERIAL	DIETZ; Allan B.;(Rochester, MN); KNUTSON; Gaylord J.;(Rochester, MN)	20160206783-A1	27 Aug 2013	United States
PLATELET PRODUCTION METHODS	Lasky; Larry C.;(Columbus, OH); Sullenbarger; Brent;(Dayton, OH); Kotov; Nicholas A.;(Ypsilanti, MI)	20100248361-A1	24 Mar 2010	United States
Method for preparing silver-loaded mesoporous silica nanoparticle carrying platelet-derived growth factor for preparing tissue engineered bone, involves placing silver-loaded mesoporous silica nanoparticles in phosphate buffer solution	MA C,CHENG X,SUN X,PU H,WIE Q,REHEMUTULA A, DENG Q	CN108371726-A	07 Aug 2018	Chinese
Microfluidic proplatelet and platelet-like particle production chamber device comprises multiple of slit channels including one or more microfluidic	MILLER W M,MCMAHON R,MARTINEZ A	WO2018237061-A1	27 Dec 2018	English

proplatelet/platelet-like particle production slits configured to expose megakaryocyte				
Synthetic platelet comprises biocompatible flexible nanoparticle including outer surface and multiple of site targeted peptides conjugated to surface and therapeutic agent, where therapeutic agent is conjugated to nanoparticle	SEN G A,PAWLOSKI C	US2019054151-A1	21 Feb 2019	English

Figure 5. Suggested design factors of platelet analogues



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