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Bio-inspired Nanomedicine Strategies for Artificial Blood Components

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Abstract

Blood is a fluid connective tissue where living cells are suspended in non-cellular liquid matrix. The cellular components of blood render gas exchange (RBCs), immune surveillance (WBCs) and hemostatic responses (platelets), and the non-cellular components (salts, proteins etc.) provide nutrition to various tissues in the body. Dysfunction and deficiencies in these blood components can lead to significant tissue morbidity and mortality. Consequently, transfusion of whole blood or its components is a clinical mainstay in the management of trauma, surgery, myelosuppression and congenital blood disorders. However, donor-derived blood products suffer from issues of shortage in supply, need for type matching, high risks of pathogenic contamination, limited portability and shelf-life, and a variety of side-effects. While robust research is being directed to resolve these issues, a parallel clinical interest has developed towards bioengineering of synthetic blood substitutes that can provide blood's functions while circumventing the above problems. Nanotechnology has provided exciting approaches to achieve this, using materials engineering strategies to create synthetic and semi-synthetic RBC substitutes for enabling oxygen transport, platelet substitutes for enabling hemostasis and WBC substitutes for enabling cell-specific immune response. Some of these approaches have further extended the application of blood cell-inspired synthetic and semi-synthetic constructs for targeted drug delivery and nanomedicine. The current article will provide a comprehensive review of the various nanotechnology approaches to design synthetic blood cells, along with a critical discussion of successes and challenges of the current state-of-art in this field.

Keywords

Bio-inspired; Nanotechnology; Nanomedicine; Synthetic; Blood; RBC; WBC; Platelet

A. Introduction

The term 'bio-inspired' refers to mimicry or adaptation of biological structures and properties in designing technologies that can simulate or leverage the function of the original biological entity. The field of nanotechnology and nano-engineering has provided exciting opportunities to achieve this at the atomic, molecular and macromolecular scales, in both

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medical and non-medical areas. In the medical field, the impact of 'bio-inspired design' is realized in the areas of cellular engineering, tissue engineering, regenerative medicine, drug delivery and nanomedicine, and a variety of materials engineering to simulate extracellular matrix components.[1-3] A unique area of bio-inspired design that has generated significant academic and clinical research interest during past 30 years is that of bio-inspired nano-scale and micro-scale engineering of synthetic (or semi-synthetic) blood cells using a variety of biomaterials-based systems. The central driving force for this research is to develop technologies that can simulate the structure and/or property of blood cells as well as plasma, to allow utilization of these technologies as 'blood substitute' when donor-derived blood is either not readily available or poses significant contamination risks.

Blood is a fluid connective tissue where living cells are suspended in non-cellular liquid matrix. The cellular components of blood render gas exchange (RBCs), immune surveillance (WBCs) and hemostatic responses (platelets), and the non-cellular components (salts, proteins etc.) provide nutrition to various tissues in the body. Figure 1A shows a schematic representation of blood circulatory path in the body, Figure 1B shows a representative histologic stain of blood smear depicting RBC, WBC and platelet, and Figure 1C-1E show cartoon depictions and representative scanning electron microscopy (SEM) images of the three blood cell types. The research focus on preserving and transporting donor-derived blood started during World War I to treat wounded soldiers, and by World War II blood transfusions became widely available. During the 1950s, multiple blood banks were established in the United States and blood donation was promoted as a form of civic responsibility. Subsequent development of many processes, e.g. freezing of RBCs, isolation of specific blood components via centrifugation and/or apheresis etc., have enhanced the utilization of whole blood and its components. Currently, whole blood as well as isolated components are clinically approved for transfusion medicine applications in civilian and battlefield trauma (e.g. in Damage Control Resuscitation), surgical settings (e.g. transplants), chronic and acute anaemias, and disease-associated, drug-induced or congenital bleeding disorders.[4-8] However, according to the Red Cross, only about 38% of US population is eligible to donate blood at any given time and only about 10% actually donate. In addition, blood-based products have somewhat limited shelf-life due to risks of pathogenic contamination. Also, RBCs and platelets upon storage develop storage lesions, which affect their stability, post-transfusion circulation lifetime and relevant bioactivities.[9,10] Currently, RBCs have a shelf-life of 20–40 days, while platelet suspensions have a shelf-life of 3-5 days, at room temperature.[11-14] Significant pre-clinical and clinical research has been directed towards enhancing the shelf-life of blood products by cold-storage, freezing, lyophilisation etc., as well as, through development of pathogen reduction technologies like psoralen-based or riboflavin-based UV irradiation, extensive serological testing of donor blood, leukoreduction and specialized storage protocols.[15-25] These approaches have indeed partially improved the shelf-life and safety of blood products, and even that partial improvement can lead to significant benefit in transfusion medicine. Blood products may also present risks of refractoriness, graft-versus-host disease, transfusion-associated immunosuppression, and acute lung injury. Also, portability of blood products, especially to remote civilian and battlefield locations, continues to be a logistical challenge. Altogether these various reasons continue to affect widespread use of blood products.[25,26] These

challenges can be potentially avoided by utilizing nano-scale and micro-scale engineering of biomaterials-based synthetic (and semi-synthetic) blood substitutes.[27-34] In fact, the interest in synthetic blood substitutes developed during the HIV crisis of the 1980s due to fear of contaminated blood products.[4] That, combined with issues of limited supply, difficulty in storage and portability, limited shelf-life, high cost, etc. have been driving the research on synthetic blood substitutes for past several decades, and several artificial blood products have undergone pre-clinical and clinical stage studies. However, currently no product is clinically approved by the FDA for human applications in the US. A 2008 meta-analysis of 16 clinical trials of five different artificial blood products suggested increased health risks in patients treated with such products, especially for RBC substitutes.[35] Although such analyses have resulted in some apprehension in clinical utility of these products, it has also directed significant emphasis on understanding and resolving the problems posed by these products at fundamental mechanistic and physiological levels. The purpose of the current article will be to provide a comprehensive review of current state-of-art in blood substitute technologies, emphasizing the role of bio-inspired nanomedicine approaches in their deign and critically discussing the successes and potential challenges in this area.

B. RBC substitutes and oxygen carrier systems

In blood, the primary function of red blood cells (RBCs) is the transport of oxygen and carbon-dioxide to and from tissues, by virtue of binding of the gases to hemoglobin (Hb) within the RBCs. The average amount of Hb in adult human RBCs (also known as Mean Corpuscular Hemoglobin, or MCH) is 27-31 picograms per cell (~250 million Hb molecules). Hemoglobin is a tetrameric protein of two α- and two β-polypeptide chains, each bound to an iron-containing heme group capable of binding one oxygen molecule. Figure 2A shows a multi-scale schematic view of RBC, Hb inside RBC, and chemical structure of iron-containing ‘heme’ group inside Hb. The binding of oxygen to Hb is positively cooperative, such that a small change in oxygen partial pressure (pO_2) can result in a large change in oxygen bound or released by Hb, resulting in a sigmoidal shape of the binding curve (Figure 2B).[36] The oxygen-carrying iron in Hb is in its reduced ‘ferrous’ (Fe^{2+}) state, which when oxidized to form methemoglobin (MetHb) with iron in the oxidized ‘ferric’ state (Fe^{3+}), is unable to bind oxygen.[37,38] Due to this reason, in natural RBCs the oxygen transport mechanism of Hb is coupled to redox cycles (e.g., driven by enzyme NAD-cytochrome b5 reductase), such that the Fe(II)-containing Hb can be maintained in its oxygen-binding state. Irreversible conversion of Hb to MetHb not only inhibits its oxygen-carrying capacity, but also leads to dysregulated vascular tone and inflammatory reactions. Furthermore, Hb in RBCs have the capability to undergo conformational changes to allow saturation loading with O_2 in the lungs (higher O_2 affinity) and then off-load O_2 in the tissue capillaries (lower O_2 affinity). Such reversible conformational control of Hb and its O_2 affinity are aided by allosteric effector molecules like 2,3-diphosphoglycerate (2,3-DPG) formed inside RBCs as a glycolytic intermediate. Therefore, engineering of RBC-inspired synthetic substitute poses a formidable challenge with regards to maintaining such oxygen-carrying thermodynamic and kinetic characteristics of Hb, maintaining the redox environment and minimizing irreversible methemoglobin formation.[39] The three main types of RBC-inspired oxygen carrier systems that have been

designed using nanotechnology approaches and have undergone extensive pre-clinical and some clinical research so far are, hemoglobin-based oxygen carriers (HBOCs), perfluorocarbon (PFC)-based emulsions, and iron (Fe^{2+})-containing porphyrins.

B.1. HBOC systems

HBOCs are essentially semi-synthetic systems since they utilize natural Hb as the oxygen-carrying component, either in chemically modified cell-free suspensions or conjugated and cross-linked with polymers along with protective enzymes, or encapsulated within biomaterials-based nano-carrier and micro-carrier vehicles.[29,40] The Hb used in these systems is usually derived from outdated human or bovine RBCs or from recombinant technologies.[40-46] When using outdated human or bovine RBCs, the Hb is isolated via cell lysis, purified by sterile filtration and chromatographic techniques and sterilized (e.g. by low heat).[47] The oxygen-binding equilibrium kinetics of Hb is positively co-operative in nature, where the Hb binds the first O_2 slowly, which in turn increases the binding affinity for the second, third, and fourth O_2 molecule to Hb, resulting in the classic sigmoidal O_2 equilibrium curve (OEC). Using such cell-free Hb suspensions presents the advantage of minimum antigenicity and the ability to transport oxygen in plasma more efficiently because of the lack of interference by cell membrane. In fact, in the early 20'th century, suspension of cell-free Hb in lactated Ringer's solution was used to intravenously treat fifteen patients, however, a large number of them were reported to develop renal toxicity and cardiovascular complications.[47] Similar results were also found in the 1950s when US Navy treated several patients with cell-free Hb.[48] Cell-free Hb was also found to have a very short circulation residence time because in cell-free environment the Hb tetramer rapidly dissociates into dimeric and monomeric forms that can bind to plasma immunoglobulins, and can undergo rapid clearance by the reticulo-endothelial system (RES) into spleen and liver, as well as renal clearance into kidneys, leading to Hb-based toxicities in these organs.[49,50] Additionally, cell-free Hb and its dissociated derivatives can also extravasate into sub-endothelial region and rapidly sequester the natural vasodilator nitric oxide (NO), resulting in conversion of NO into nitrate (dioxygenation reaction) and oxy-Hb to Met-Hb.[51] This NO-scavenging results in vasoconstriction and cardiovascular complications. Furthermore, the absence of the allosteric effector molecule 2,3-DPG in cell-free Hb can lead to unnaturally high oxygen affinity, making oxygen off-loading problematic. Cell-free Hb can also change blood osmolarity, leading to alteration of blood volumes and associated side-effects. Altogether, these reasons have resulted in cell-free human Hb being deemed problematic for oxygen-carrying applications. Instead of human Hb, studies have also been conducted with bovine Hb, but this also presents similar issues of stability, extravasation, NO-scavenging and renal clearance issues. One interesting way to address many of these issues is by development of recombinant Hb (e.g. in E. Coli) where specific mutations can enable decrease in dissociation and reduction in NO-binding capacities, but the correct combination of mutations that can lead to an ideal Hb design is still not completely known.[52-54] Recombinant technologies are also substantially expensive. Therefore, a significant volume of research has been focused on molecular stabilization and functional modulation of Hb utilizing chemical modifications like cross-linking, polymerization and macromeric surface-conjugations. The goals of these modifications are to reduce Hb

dissociation, extravasation and renal clearance, while maintaining reasonable circulation life-time and oxygen loading/off-loading capacities.

B.1.1. Chemically modified HBOCs—Chemical cross-links in Hb can be created both intra- and intermolecularly. For example, Hb can be cross-linked intramolecularly between its two α-subunits using acylation with bis-(3,5 dibromosalicyl)-fumarate (also known as Diaspirin) and using this strategy on human Hb led to a product called HemAssist from Baxter, USA.[40,55,56] This product showed an increase in circulation time up to 12 h compared to <6 h for unmodified Hb, but the cross-linked Hb unfortunately showed a 72% increase in mortality rates in human patients compared to saline, and clinical trials were discontinued.[57] An analogous crosslinking approach between the α-subunits of recombinant Hb using Glycine as the crosslinking agent led to a product called Optro from Somatogen, USA, which also resulted in risks of cardiac arrest and mortality.[58-60] Instead of site-specific intramolecular crosslinking only, polymerized Hb was created using bifunctional cross-linking reagents like glutaraldehyde (e.g. the products Hemopure from Biopure, USA and PolyHeme from Northfield Labs, USA) and o-raffinose (e.g. the product HemoLink from Hemosol, Canada).[61,62] One challenge in these approaches is to precisely control polymer molecular weight and rigorous purification steps are necessary to ensure product quality. PolyHeme was reported to progress into Phase III clinical trials in the US in treating trauma-associated blood loss and showed a decreased need of natural blood transfusions.[60] Clinical trials with HemoPure also showed a reduced need of additional blood transfusions in cardiac surgery.[63] Although HemoPure has not been approved by the FDA in the US, it has been reported to have clinical approval in South Africa for acutely anaemic patients. HemoLink also advanced to phase III clinical trials but was discontinued in 2003 when patients receiving treatment experienced adverse cardiac events. In fact, all of these products in their clinical trials have shown high risks of transient hypertension, organ damage through micro-vascular constriction and dysfunction, gastro-intestinal distress, nephrotoxicity, neurotoxicity, and increased mortality.[64,65] Instead of intramolecular cross-linking and intermolecular polymerization, modification of Hb has also been carried out with macromeric bioconjugation to increase stability and vascular residence time while reducing immune recognition.[66-68] Important examples of this approach are found in polyethylene glycol (PEG) modification of Hb (e.g. the products Hemospan from Sangart Inc., USA and PEG-Hb from Enzon, USA) and poly(oxyethylene) modification of pyridoxylated crosslinked Hb (e.g. the product PHP from Apex Bioscience, USA). PEG-ylated Hb products have undergone extensive clinical trials and the studies showed risks of bradycardia and elevation of hepatic pancreatic enzymes even at low doses.[69] Nonetheless, the Phase I and Phase II clinical trials showed that Hemospan was well-tolerated in humans for efficient oxygen delivery, and Phase III trials in orthopedic surgery patients were carried out in Europe. The trials suggested that the risk of cardiovascular and renal dysfunctions still persisted with such chemically modified Hb products.[70] Products like PHP have also indicated such risks of cardiovascular and renal dysfunctions. During the past two decades it has been identified that cell-free Hb (including chemically modified versions) are potent scavengers of nitric oxide (NO) via rapid irreversible binding (rate constant ~ $10^7 M^{-1}s^{-1}$), which in turn affects systemic and pulmonary vascular tone, resulting in vasoconstriction, hypertension, and lowering of cardiac output.[71,72] A resolution of this issue has been

attempted by modifying Hb molecule into becoming an NO carrier through S-nitrosylation of cysteine residues in the β-subunits of Hb or imparting the ability of enzymatic transformation of Hb into a source NO donor in presence of nitrites, but with limited success in vivo.[73] Natural RBCs contain enzymes like catalase (CAT) and superoxide dismutase (SOD) that help mitigate the oxidative stresses stemming from superoxide moieties in injured and ischemic tissues. Based on this rationale, in an interesting approach these enzymes have been crosslinked to polymerized Hb to form PolyHb-SOD-CAT, which showed combined advantages of long circulation time and reduced oxidative damage.[74,75] Another interesting approach is to incorporate regulatory molecules such as 2,3-DPG and methemoglobin reductase along with Hb in appropriate HBOC systems, to prevent hemoglobin oxidation. In another recent approach, a product named HemoTech has been developed that uses purified bovine Hb cross-linked intramolecularly with ATP and intermolecularly with adenosine, and conjugated with reduced glutathione (GSH).[76] The novelty of this design is the use of pharmacologically active molecules (ATP, adenosine and GSH) as the chemical modifiers, where ATP regulates vascular tone through purinargic receptors, adenosine counteracts the vasoconstrictive properties of Hb via stimulation of adenosine receptors, and GSH shields the ‘heme’ from NO and reactive oxygen species. The preclinical and early phase clinical studies have shown that HemoTech works as an effective oxygen carrier in treating blood loss, anaemia and ischemic vascular conditions, and further studies are expected. In another recent nanotechnology approach, core-shell cluster structures were formed by conjugating human serum albumin (HSA) on Hb using Hb surface lysines conjugated to HSA cysteine-34 using α-succinimidyl-ε-maleimide cross-linker.[77] These Hb-HSA clusters are envisaged to have low risks of rapid clearance and extravasation, and high circulation stability. A further modification of these Hb-HSA core-shell nanoclusters was recently reported where anti-oxidant enzymes and platinum nanoparticles were embedded in the HAS pockets for protection of Hb.[78] These nanocluster designs have only been evaluated for their oxygen-binding capacity, redox properties and stability in vitro, and rigorous in vivo studies would be needed to establish their in vivo applicability. Figure 3 shows some of the prominent designs based on chemical modification of cell-free Hb. In spite of promising pre-clinical and some clinical results, most of the chemically modified Hb products have been withdrawn from clinical studies and discontinued in production, due to substantial clinical data indicating more risks than benefit, owing to chemical heterogeneity of final product, variable stability, sub-optimal vascular residence time, non-ideal oxygen loading/off-loading capabilities, rapid irreversible conversion to methemoglobin, and increased cardiovascular and renal dysfunction issues. While some of the newer products are refining their design and processing to address these issues, a parallel direction of the research and development has focused on encapsulation of Hb within various micro- and nano-carrier vehicles.

B.1.2. Encapsulated HBOC systems—The advent of microparticulate and nanoparticulate technologies have revolutionized the delivery of pharmaceutical compounds, by encapsulating the compounds within such particulate vehicles that can protect the compounds from plasma-induced effects, increase the circulation time of the compounds and allow sustained delivery to cells, tissues and organs. Naturally, this concept was also adapted to create HBOCs that encapsulate Hb within suitable particulate carrier vehicles. In fact, the

concept and demonstration of bio-inspired artificial cells was presented as early as the 1950s and 1960s by Chang and colleagues, by encapsulating Hb as well as other proteins and enzymes within microvesicles bound by polymeric membranes. The membrane material originally used was collodion (cellulose nitrate) and later changed to biodegradable polyethylene glycol-polylactide (PEG-PLA).[79,80] These 'hemoglobin corpuscles' showed oxygen equilibrium curves similar to RBCs and retained activity of RBC-relevant enzymes like 2,3-diphosphoglycerate (2,3-DPG), carbonic anhydrase and CAT.[79-81] However, a principal challenge was posed by the rapid macrophagic removal of these micron-sized polymeric vesicles from circulation, resulting in very short vascular residence time. Reducing the diameter to ~1 micron only marginally improved the circulation, and a significant research effort was directed towards further improving the vascular residence time by modifying the surface of the polymeric vesicles with lipids and polysaccharides. In an analogous approach, Djordjevich et al reported on encapsulation of Hb in micron and sub-micron size lipid vesicles (liposome-encapsulated Hb or LEH), with membrane made of phospholipids and cholesterol.[31,82-84] This design was to essentially mimic the physiological state of Hb in RBCs where it is encased within the cell membrane in a protected environment that preserved the suitable redox mechanisms for Hb function. A number of variations of this design followed, e.g., 'neohemocytes', 'TRM-645 Neo Red Cells' etc., where the primary focus was to maintain uniform Hb-encapsulation levels and uniform size distribution of the vesicles, prevent vesicle destabilization or fusion over time, and thereby enhance vesicle stability upon storage while maintaining the RBC-mimetic oxygen transport properties of the encapsulated Hb.[85-87] During the 1990s the 'Stealth Liposome' technology was established, where nanoscale lipid vesicles (100-200 nm in diameter) were surface-functionalized with polyethylene glycol (PEG) to decrease fusion and destabilization upon storage, reduce opsonization and prevent rapid macrophagic uptake, and this design significantly enhanced the circulation residence time.[88,89] Consequently, this technology was adapted to form Hb-encapsulated PEG-ylated liposomal vesicles (HbV).[90-92] For HbV preparation, 1,2-dioctadecadienoyl-sn-glycero-3-phosphatidylcholine (DODPC) was used as the major membrane phospholipid, such that γ-irradiation induced radiolysis of water molecules in the vesicles generated hydroxy (OH) radicals that promoted intermolecular polymerization of dienoyl groups in DODPC to produce remarkably stable liposomes that could withstand freeze-thawing, freeze-drying, and rehydration processes. The incorporation of Hb in PEG-ylated sub-micron sized stealth liposome vesicles resulted in substantial improvement of circulation life-time (~60 hrs in some animal models) and many refinements of this approach have been reported during the past two decades regarding HbVs.[93-96] Incorporation of Hb within lipidic vesicles are analogous to incorporation within cell membranes, and therefore the oxygen transportation ability of HbVs were partly similar to natural RBCs, with comparable oxygen saturation and release kinetics. Also, the liposomal encapsulation of Hb reduces its scavenging effect on NO and thereby reduces the subsequent negative effects on vasculature. The encapsulation also prevents glomerular clearance of Hb and thereby reduces nephrotoxicity. The current optimized HbV product contains about 30,000 Hb molecules encapsulated within one PEG-ylated liposomal vesicle of ~250 nm in diameter.[31,91] In comparison, a natural RBC is ~7μ in diameter and ~2μ in thickness, containing about 250 million Hb molecules. The HbVs have undergone extensive research in pre-clinical animal models for potential use as a synthetic RBC substitute in transfusion and

resuscitative approaches in perioperative settings, massive hemorrhagic shock and hemodilution incidents, and oxygenation of ischemic as well as transplanted tissues and organs.[31,91-95] These studies show significant promise of HbVs as semi-synthetic RBC mimics, however, these systems can still present issues of broad size distribution of the vehicles, variations in Hb-encapsulation efficiencies, variable pharmacokinetics and complement-mediated immune response in vivo. Further research is currently being directed towards resolving these issues for potential clinical translation of HbV designs as a synthetic RBC substitutes.[31,96-98] Interestingly, instead of encapsulating Hb, some research approaches have also attempted to encapsulate oxygen (O_2) directly within phospholipid microvesicles (2-4 μ in diameter) to deliver O_2 to deoxygenated RBCs in circulation.[99,100] Although these oxygen-loaded microbubbles were found to be stable for a few weeks in storage with only small extent of oxygen loss, in vivo they have a very short circulation life-time (< 1hr). Therefore, treatment with these systems would require multiple or repeated dosing, which may then lead to negative effects of dysregulated oxidative stress and associated immune response. Therefore, long-term safety profile of this technology needs to be rigorously evaluated. Encapsulation of Hb has also been studied in other microparticle and nanoparticle systems besides using liposomal vesicles. In pioneering work by Chang et al, Hb has also been encapsulated within nanoscale polymeric particles made from PEG-PLA and analogous block copolymers.[101,102] These polymeric nanoparticles, about 80-200 nm in diameter, can allow oxygen transport kinetics of encapsulated Hb at ranges similar to natural RBCs and the polymeric material is biocompatible and biodegradable. Enzymes that maintain the redox environment for stability and O_2/CO_2 transport of Hb (e.g. carbonic anhydrase, CAT, SOD, MetHb reductase etc.) can also be encapsulated within the same nanoparticles to further refine their function towards mimicking RBC action.[103] This concept has also been adopted for other polymer systems like poly(ε-caprolactone)/poly(L-lactic acid) (PCL/PLA) copolymers, poly(L-lysine) (PLL), poly(lactic-co-glycolic acid) (PLGA)/PEG copolymers etc.[104,105] Amphiphilic block-copolymer systems also provide the ideal building blocks for designing polymer vesicles, otherwise known as polymersomes, analogous to liposomes. These polymersome systems have also been recently utilized to load Hb, to create polymerosome-encapsulated Hb (PEH).[106] The Hb loading in these PEH systems has been reported to be 1-2 mg/ml, compared to human blood (i.e. within RBC) concentration of ~150mg/ml. Utilization of hollow fiber membrane module based extrusion system can provide an interesting way to manufacture these PEH systems.[107] These PEH systems can encapsulate both bovine and human Hb, and can show oxygen binding and release equilibrium kinetics and other biophysical parameters similar to RBCs. This indicates considerable promise towards the application of PEH systems as a synthetic RBC substitute in transfusion medicine, but currently very limited in vivo evaluation data is available for these systems. A potential issue with polymersome systems may be their higher shell thickness compared to liposomes, which can lead to longer diffusion time for oxygen to saturate the encapsulated Hb or to be released from the Hb to tissues. Modulation of polymer molecular weight of the shell and therefore of the shell-thickness can provide a way to influence oxygen transport from PEH systems. The higher stability of polymersomes compared to liposomes, both in storage and in vivo, may provide additional advantages for its use as Hb-encapsulated artificial RBC systems. Hb has also been encapsulated in microparticles by its co-precipitation with calcium carbonate ($CaCO_3$), followed by

crosslinking with glutaraldehyde and selective dissolution of $CaCO_3$, with Hb quantity per microparticle close to natural RBCs.[108] These Hb-microparticles have shown oxygen binding and release characteristics similar to free Hb, but much enhanced circulation lifetime compared to free Hb. Analogous Hb microparticles carrying about 80% Hb content compared to natural RBCs, have been recently reported where Hb and $MnCO_3$ were co-precipitated, immediately followed by human serum albumin addition for encapsulation and stabilization of the particles.[109] These particles have shown limited NO scavenging and reduced effect on vasoconstriction. Recent reports have also demonstrated the capability of covalently conjugating Hb directly to the hydrophobic or hydrophilic domain of block-copolymers and then micellize the resultant conjugates to form Hb-loaded micelles.[110,111] In another interesting nanotechnology approach, $MnCO_3$ nanoparticles were used as templates to deposit layer-by-layer (L-B-L) assemblies of Hb and dialdehyde heparin (DHP), followed by crosslinking to stabilize the layers and selective dissolution of the template core.[112] A similar approach was also used to form L-B-L coated nanotubes where alternate layers of Hb, DHP and the enzyme catalase (CAT) were deposited, to create systems for potential application in treating oxidative stress.[113] These complex nanoscale structures have been characterized in vitro for their morphology, stability, cytotoxicity and in some cases biofunctionality, but no pre-clinical evaluation for oxygen carrying efficacy in vivo, has been reported yet. Figure 4 shows some representative designs and components for encapsulated Hb systems that have undergone and are currently still undergoing in vitro and in vivo evaluation for RBC-mimetic oxygen carrier application.

Some Hb-encapsulation approaches have focused on the physico-mechanical properties of natural RBCs that significantly influence their biological functions. Natural healthy RBCs are biconcave discoid in morphology, with a diameter of ~8 μm and a thickness of ~2 μm, and are highly flexible (Young's Modulus 0.1-0.2 kPa), that enables them to change their shape and morphology when passing through microvascular circulation.[114,115] The mechanical integrity and viscoelastic nature of RBCs during their cyclical deformation is maintained by a two-dimensional spectrin network attached to the cytosolic side of their membrane. Oxygenated Hb renders RBCs significantly more deformable than deoxygenated Hb, and this enables the deformation of RBCs to transport oxygen in the microvasculature. The size shape and flexibility of RBCs are also known to influence their hemodynamic distribution in the blood flow field, where in mid to large vessels the RBCs flow in the center of the parabolic flow field, while in small vessels and capillaries RBCs can become distributed throughout for efficient oxygen exchange.[116] These considerations have recently led to biomaterials-based micro- and nanoscale mimicry of RBC-relevant size, shape and flexibility attributes into Hb-encapsulating synthetic constructs. For example, polyelectrolyte driven layer-by-layer assembly has been used to create polymeric particles that mimic the shape and deformability of natural RBCs.[117] In this design, Hb and BSA were electrostatically deposited on the surface of RBC-shaped PLGA particles of ~7 μm diameter and 400 nm shell thickness, and then the PLGA core was selectively dissolved to yield RBC-shaped Hb-loaded particles capable of high elastic deformation. Similar RBC-mimetic particles have been fabricated using PEG hydrogel system in a stop-flow-lithography (SFL) approach where the mechanical properties of resultant particles could be controlled by modulating cross-linking density of the hydrogel systems.[118] In a different approach, RBC-

mimetic particles were fabricated from acrylate hydrogels using a ‘particle replication in nonwetting templates’ (PRINT®) technology.[119] These particles were made in 2–3 μm molds, such that, upon hydration, the particles swelled to disks with diameters between 5.2-5.9 μm and heights between 1.22-1.54 μm. Filling of the molds resulted in a meniscus, leaving the particles thinner in the middle and thicker at the edges, resembling the biconcave nature of RBCs. All of these RBC-mimetic particle designs have demonstrated elastic deformation capabilities in vitro for transport through narrow channels, and in vivo they have shown variable circulation lifetime depending on their elastic modulus. Although research approaches using these particles have reported Hb encapsulation via physical trapping or covalent tethers (e.g. via ethyl(dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry), detailed oxygen transport capabilities and in vivo transfusion medicine potential have not been explored or reported in detail. In another recent approach, liposome-encapsulated actin-hemoglobin (LEAcHb) synthetic RBCs with natural RBC-mimetic shape properties were prepared using a polymerized actin core.[120] Although these particles were much smaller (~136.8 nm) than RBCs, the biconcave shape along with the mechanical support of the membrane improved the half-life from ~8 h to >72 h. In natural RBCs, the negative surface charge electrostatically prevents RBC aggregation over a distance of 20 nm and this rationale has led to some research in mimicking RBC-relevant surface charge on Hb-encapsulating PEG–PLA nanoparticles (<200 nm in diameter) using cetyltrimethylammonium bromide (CTAB) or anionic sodium dodecyl sulfate (SDS) surfactants.[121] Cationized particles were found to have a half-life of ~11 hrs (8-fold higher than untreated particles), while the anionized particles were quickly eliminated, giving a half-life of <1 hr. In yet another recent approach, a novel amphiphilic polymeric system was developed using polyethylene imine (PEI) modified with palmitic acid and was used to form toroidal shaped nanoparticles (termed nanobialys, ~200 nm diameter) that can encapsulate Hb, as well as, maintain redox enzymatic environment for Hb activity by co-encapsulation of 2,3-DPG and leuko methylene blue.[122] These novel Hb-containing particles, termed Erythromer, have shown some promise of oxygen transport in vivo, and detailed biocompatibility studies (e.g. for PEI which can pose cytotoxicity issues), circulation lifetime and stability, Hb-loading capacity and oxygen transport capabilities etc. would need to be further evaluated to establish their clinical potential as synthetic RBC substitute in transfusion medicine. It is interesting to note that a hypothetical bio-inspired concept of microparticulate ‘artificial red blood cells’ termed *Respirocyte* was proposed almost two decades ago by Robert A. Freitas Jr in his paper "A Mechanical Artificial Red Blood Cell: Exploratory Design in Medical Nanotechnology", but an ideal Hb-loaded synthetic RBC substitute for in vivo transfusion applications still remains to be achieved and clinically approved.[123] A high volume of research continues to be directed in this area in the US and globally, that may ultimately lead to a viable RBC substitute.

B.2. Perfluorocarbon (PFC)-based oxygen carrier systems

Due to the issues of stability, circulation lifetime, potential NO-scavenging properties and toxicity of Hb-based oxygen-carrying nanotechnologies, a parallel research interest has developed in in non-Hb based fully synthetic macromolecular (polymeric) systems capable of transporting oxygen. To this end, the class of macromolecules that have undergone the most robust volume or research and translational activities is that of perfluorocarbons

(PFCs). PFCs are uniquely both hydrophobic and lipophobic which contributes to their biological inertness, and this property has resulted in utilization of liquid PFCs in magnetic resonance imaging and gas-filled PFC microbubbles in ultrasound imaging contrast agents in biomedical diagnostic applications.[124-127] PFCs have the ability to dissolve oxygen physically, where O_2 is loosely bound by van der Waals type interaction between PFC macromers, and not chemically bonded as they usually are with Hb. This dissolution equilibrium of O_2 in PFCs follows Henry's law (i.e. oxygen partial pressure), resulting in a linear oxygen binding/transport profile, in contrast to the sigmoid co-operative binding profile found for oxygen binding to Hb.[128,129] Figure 5 shows chemical structure of some representative PFCs that have been investigated extensively for oxygen transport applications, as well as, the linear profile of O_2 binding to PFCs compared to the non-linear exponential profile of O_2 binding to Hb or HBOCs. PFCs are biologically inert liquids and gases, characterized by strong intramolecular and weak intermolecular C-F and C-C bonds. This essentially means that a much higher oxygen partial pressure is required to ensure sufficient dissolution and transport of oxygen by PFCs, compared to that by Hb. On the other hand, due to the linear profile, oxygen off-loading capacity is better for PFCs compared to Hb. Due to immiscibility of PFCs in water, intravascular application of PFCs for oxygen carrying application requires rigorous emulsification of PFCs with suitable surfactants (e.g. phospholipids) and the oxygen solubility in PFC emulsions is independent of the surfactant used.[130] Several PFC-based emulsions have been investigated extensively as oxygen carriers in preclinical and clinical stages.[118-121] Fluosol-DA (Green Cross Corp, Japan), an emulsion of 10-20% perfluorodecalin in albumin, was extensively tested in pre-clinical animal models and was the first PFC system to progress into clinical trials and to receive FDA approval in 1989. However, this product was soon recalled because of issues like low oxygen transport capacity (0.4 mL oxygen/100 mL compared to blood's reported oxygen transport capacity of 20.1 mL/100 mL), premature oxygen release, short shelf-life, temperature instability, slow excretion and serious side-effects.[131] Fluosol was also implicated in reducing platelet counts (thrombocytopenia), febrile symptoms and macrophage activation (inflammation trigger). The limitations of Fluosol PFC systems were significantly resolved by newer generation PFC compounds like perfluoroctyl bromide (also known as perflubron), perfluorodecyl bromide and perfluorodichlorooctane.[132-138] Using these compounds in specific ratios along with egg-yolk phospholipids, a PFC-based oxygen carrier named Oxygent (Alliance Pharmaceutical Corp, U.S.A.) was developed where the droplet size could be maintained at 160-180 nm in diameter, that showed reduced macrophage activation.[135,136] Intravascular circulation half-life for Oxygent droplets was found to be ~10 hr for a dose of 1.8 g/kg in humans.[138,139] Oxygent was found to render higher oxygen delivery compared to Fluosol, but was still ~30% less efficient than natural RBCs. Another analogous PFC product was developed using perfluorodicholorooctane, egg yolk phospholipid and triglyceride, named Oxyfluor (HemaGen, U.S.A.), with droplet diameters in the range of 220-250 nm.[137] This product has also shown similar delay in macrophagic uptake and therefore similar circulation residence time as Oxygent. Due to nanoscale diameter, these perfluorocarbon droplets can occupy small plasma volumes and can circulate through microcapillaries, thereby providing significant ability of oxygen delivery in microcirculation. In preclinical models in vivo, both Oxygent and Oxyfluor have shown promising benefit in oxygen delivery, although both products have also shown some

platelet-depleting (thrombocytopenic) effects.[140] In several large animal (e.g. canine) models of hemodilution and cardiopulmonary bypass, these PFC systems have shown the ability to increase oxygen transport and metabolism.[141] In fatal hemorrhagic models these oxygen carriers could improve resuscitation and reduce mortality significantly. These promising results have led to clinical evaluation of such products in human patients undergoing major non-cardiac surgery, hemodilution and hemorrhage.[142-144] In such scenarios Oxygent was evaluated as a substitute for autologous blood transfusion and this product was able to significantly transport oxygen and reverse transfusion triggers. These results indicated that such PFC based oxygen carrier systems are at least as effective as autologous blood to reverse transfusion triggers and therefore can act as RBC substitutes to some extent. However, advanced clinical trials of such products in patients undergoing cardiopulmonary bypass were terminated in the U.S. because of increased risk of stroke and adverse events, although deeper analysis of the data has suggested that these adverse events were associated more with the study protocol rather than the PFC product itself.[139] Oxycyte (Oxygen Biotherapeutics Inc, U.S.A.), is a third generation PFC emulsion containing an aqueous 60% emulsion of perfluoro-tert-butylcyclohexane with purified egg yolk phospholipids, that has undergone pre-clinical trials in several animal models and some clinical trials in patients with traumatic brain and spinal chord injury.[145-147] Although some safety concerns were raised regarding Oxycyte's effect on the immune system and possible hemorrhagic side effects, recent animal studies have alleviated FDA concerns and studies are under way. Perftoran (Perftoran, Russia) is another PFC emulsion of perfluorodecalin and perfluoromethylcyclopiperidine in a nonionic surfactant that has been widely investigated in Russia.[148,149] Although this product has shown adverse effects such as hypotension and pulmonary complications, randomized clinical trials with Perftoran conducted in Mexico City on patients undergoing cardiac valvuloplasty showed higher intraoperative pO_2 levels, and reduced need for allogenic RBCs. Further clinical studies are expected to establish the benefit of this product.

B.3. Synthetic porphyrin systems

The oxygen-transporting active site of natural hemoglobin is the 'heme' part, which consists of a porphyrin tetrapyrrole ring surrounding an iron atom (Figure 2A). Based on this, researchers have developed Fe(II)-bearing porphyrin systems for oxygen transport.[150,151] To this end, "picket fence" Fe^{2+} porphyrin molecules were created that demonstrated that reversible oxygenation of myoglobin and hemoglobin is possible via immobilization of the five-coordinate ferrous 'heme' in a sterically hindered hydrophobic matrix.[152] These systems showed cooperative oxygen binding similar to natural Hb, but were prone to irreversible oxidation in aqueous media. To resolve this issue, a hydrophobic environment was created for these molecules, for example, using liposomes, where amphiphilic Fe^{2+}-porphyrin bearing four alkylphosphocholine groups were efficiently embedded into the bilayer of phospholipid vesicles (e.g., LipidHeme).[153,154] The resultant vesicles demonstrated reversible binding and release of oxygen similar to natural Hb. Instead of lipid vesicles, human serum albumin particles have also been used to incorporate these synthetic Fe^{2+} porphyrin systems, and these have shown oxygen-transporting efficiency similar to RBCs. Surface modification of these particles with PEG have resulted in increased circulation time and reduced oxidation. Another porphyrin-based interesting design is

HemoCD, that is comprised of a 1:1 complex of 5,10,15,20-tetrakis (4-sulfonatophenyl) porphinatoiron(II) (Fe[II] TPPS) and a per-*O*-methylated β-cyclodextrin dimer having a pyridine linker (termed HemoCD or Py3CD).[155] This system showed oxygen affinities similar to natural Hb and was found to be gradually autoxidized in aqueous environment via nucleophilic attack of water molecule to the O2–Fe bond. The circulation stability of these systems could be further enhanced by surface-decoration with PEG-based dendrimers.[156] Figure 6 shows representative design schematic and nanomaterials used for the various porphyrin-based oxygen carrier systems. The in vivo application and clinical promise of these nanoengineered porphyrin systems as oxygen carriers need to be further evaluated in appropriate pre-clinical animal models.

C. Platelet substitutes, synthetic hemostats and platelet-inspired drug delivery systems

Platelets are anucleated blood cells primarily responsible for blood clotting and hemostasis. Platelets are produced from mature megakaryocytes and under hemodynamic blood flow environment they have the ability to undergo margination towards the blood vessel wall while the RBCs congregate more towards the centre of the blood vessel, in a parabolic flow profile.[157-159] By virtue of this, platelets can stay in constant surveillance of the vessel wall and can rapidly provide hemostatic respond where needed. As depicted in Figure 7, platelet's mechanisms for hemostatic response at a vessel bleeding site is mediated by (i) rapid adhesion of platelets at the site by binding to specific proteins like von Willebrand Factor (vWF) and collagen that are exposed at the site, (ii) activation and rapid aggregation of platelets at the site via inter-platelet bridging by blood protein fibrinogen (Fg) interacting with platelet surface integrin GPIIb-IIIa (primary hemostasis), and (iii) facilitation of coagulation cascade mechanisms on the negatively charged phosphoserine-rich membrane surface of active platelets aggregated at the site.[160,161] Loss of platelets due to trauma or surgery, as well as, drug-induced or congenital defects in platelet number and function, can lead to a variety of bleeding complications, and transfusion of natural platelets are routinely used in the clinical treatment of such conditions.[162-165] These transfusions primarily use allogeneic platelet concentrates (PCs) suspended in autologous plasma, stored at room temperature and these PCs have a high risk of pathologic (mostly bacterial) contamination, resulting in very short shelf-life (~3-5 days).[165,166] Pathogen reduction technologies (e.g. psoralen-based UV treatment) can reduce contamination risks, but so far these have extended the PC shelf-life only marginally.[166-169] Several other platelet-derived products are currently under investigation, e.g., frozen (−80°C) platelets, cold-stored (4°C) platelets, lyophilized platelets, platelet-derived microparticles and infusible platelet membranes (IPM), and trehalose-stabilized platelets (Thrombosomes, CellPhire, USA), with a vision to extend shelf-life of platelet-based hemostatic products without compromising biofunctional properties.[170-173] PCs and natural platelet-derived products are usually obtained from pooled blood of multiple donors, and this presents significant risks of blood-borne infection that, in turn, necessitates rigorous serological testing and expensive leukoreduction techniques.[23,174] Due to these various issues with natural platelet-based systems, a parallel significant interest has emerged in synthetic platelet substitutes that can render efficient hemostasis while allowing advantages of large-scale preparation, minimum contamination risk via effective

sterilization, longer shelf-life, no need for blood type matching and reduced risks of biologic or pathologic side effects.[30,34,35,175]

To this end, a variety of platelet-inspired systems have focused on mimicking platelet's biochemical mechanisms of injury site-specific adhesion, aggregation, and coagulation promotion via surface-modification of microparticle and nanoparticle systems with platelet surface-relevant glycoproteins and ligands. It is to be noted here that platelets are micro-scale (2-3 μ diameter) discoid particles that have a large variety of surface glycoproteins and receptors present at different densities. For example, platelet surface integrin αIIbβ3 (GPIIbIIIa) that binds to the ligand fibrinogen (Fg) is present at ~80,000 copies per platelet, surface glycoprotein GPIb-IX-V that participates in binding to von Willebrand Factor (vWF) is present at ~50,000 copies on the surface, and surface protein α2β1 (GPIaIIa) that binds to collagen is present at ~3000 copies per platelet.[176] Besides these, there are multiple other surface proteins (e.g. cell adhesion molecules or CAMs, thrombin receptors, ADP-receptors, selectins, tetraspanins, α5β1 and α6β1 integrins etc.) that are present at densities of tens to hundreds to thousands. In simulating the major hemostatic functions and activity of platelets on semi-synthetic or synthetic nanotechnology platforms, the focus has not necessarily been to simulate the exact densities of interactions but rather the type of interactions. For designs that have directly used platelet-derived membranes for vesicle fabrication or nanoparticle coating, the surface-density of interactive motifs is dependent upon whatever bioactive surface proteins remain in the membrane post-isolation and processing. In contrast, for fully synthetic designs the number of ligands per unit surface area per particle can be theoretically estimated based on dimension of particles and concentration (e.g. mole %) of ligands reacted with particles, and that number can very from hundreds to thousands per particle. Furthermore, since platelet's physico-mechanical properties (e.g. shape, size, flexibility etc.) can influence their margination behaviour under hemodynamic flow environment, some recent research approaches have also been directed towards leveraging platelet's biophysical parameters in designing nanoparticle and microparticle based 'synthetic platelet' systems. In another interesting approach, conventional drug delivery particles have been coated with platelet-derived membranes, to impart bioinspired targeting properties. Figure 8 shows some representative designs and components for platelet-inspired nanomedicine systems for hemostatic applications as platelet substitutes and the following sections describe the approach and functional promise of these designs. It is to be noted that in parallel to leveraging and mimicking platelet's hemostatic mechanisms, a subset of 'synthetic hemostat' research has also focused on development and utilization of body's coagulation factor components and coagulation cascade outputs as injectable natural, semi-synthetic or synthetic molecules to augment hemostasis. Classic examples of this are the development of recombinant coagulation factors, a variety of natural and synthetic biomaterials that induce activation of platelets and/or coagulation, peptide-modified synthetic polymers that mimic and amplify mechanisms of fibrin strengthening and drug delivery platforms that can deliver pro-coagulant molecules at bleeding sites.[177-186] These systems are not necessarily 'synthetic blood substitutes', but they form an important area of biomaterials and nanotechnology research in terms of modulating blood clotting properties, with potential applications in treating hemorrhagic complications and promoting wound healing. Detailed

discussions of these systems are beyond the scope of the current review, but several informative reviews on many of these systems are available elsewhere.[179,187-189]

C.1. Nanomedicine systems inspired by platelet adhesion mechanisms

Natural platelets adhere to the injury site via several specific mechanisms. Upon vascular injury, the injured endothelial cells lining the luminal wall of blood vessels secrete von Willebrand Factor (vWF) molecules from their Weibel Palade bodies.[190] The injury site also has exposed sub-endothelial collagen. The vWF molecule is usually secreted as a globular protein, which unravels under hemodynamic shear forces at the injury site and can multimerize on injured endothelium and exposed collagen.[191] The unraveled vWF exposes the A1-domain, which has binding specificity to platelet surface receptor component GPIbα of the GPIb-IX-V complex. This vWF-to-platelet GPIbα interaction is shear-dependent and reversible, and leads to platelet attachment and rolling at the injury site.[191,192] Concomitantly, platelet surface glycoproteins GPIa-IIa and GPVI, with binding capability to collagen, can anchor onto the exposed collagen at the injury site and this synergistically strengthens the adhesion of platelets at the site.[192] The subsequent platelet-mediated mechanisms of coagulation and hemostasis happen in tandem such adhesion events. Therefore, inspired by these adhesion mechanisms, nanomedicine research has focused on developing surface-engineered particulate systems that simulate platelet's vWF-binding and collagen-binding capabilities. One of the earliest designs in tis aspect was 'plateletosomes', that involved isolation of platelet membrane glycoproteins via detergent-based extraction techniques and their subsequent incorporation within the lipid membrane of liposomes.[193] While this approach ensures retention of platelet's own biofunctional surface proteins, the extraction, purification, sterilization and liposome membrane incorporation make this strategy potentially complex and expensive to scale up. These complex issues could be avoided by utilizing recombinant technologies, where recombinant GPIbα (rGPIbα that binds to vWF's A1 domain) and GPIa-IIa (rGPIa-IIa, that binds to collagen) could be conjugated on the surface of liposomes, latex beads or albumin-based particles to create synthetic systems that simulate platelet's injury site-adhesive capabilities.[194,195] In further advancement of this approach, both rGPIbα and rGPIa-IIa have been 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, thereby closely mimicking natural platelet adhesion.[196]

Recombinant technology approaches to make analogous platelet surface protein can be quite expensive in the context of scaling up and clinical translation. Also, the large size of these recombinant protein fragments can lead to issues of steric hindrance regarding their combinatorial decoration on the surface of nano- and microparticles. These issues can be potentially addressed by using small molecular weight peptides that have vWF-binding and collagen-binding capabilities. An early example of this is found in reports focusing on the development of GPIbα-relevant 15-mer peptides that have binding capability to vWF.[197] These peptides were tethered onto liposomal surfaces stabilized via cross-linking, and the resultant liposomal constructs, 150-200 nm in diameter, have been tested as 'synthetic platelet substitutes'.[198] Although the biochemical characterizations of these constructs have been reported, the actual hemostatic efficacy evaluation of these constructs in vitro and in

vivo have not been demonstrated in any pre-clinical animal model. In a more recent approach, researchers have utilized a vWF-binding peptide (VBP) sequence TRYLRIHPQSWVHQI derived from the C2 domain (residues 2303-2332) of the coagulation factor FVIII and a collagen-binding peptide (CBP), which is a 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 affinity to platelet collagen receptors GPIa/IIa and GPVI (i.e. minimal risk of systemically activating platelets), to mimic platelet adhesion mechanisms on nanoparticles.[199,200] Using liposomes as model nanoparticles, VBPs and CBPs were conjugated onto the particle surface. Liposomes decorated with only the VBP moieties exhibited enhanced adhesion onto vWF-coated surfaces or on collagen surfaces in presence of soluble vWF, under high shear flow. Studies also indicated that this vWF-binding mechanism of VBP is possibly occurring at the D'-D3 domain of vWF and not the platelet GPIbα-interactive A1 domain.[200] These studies suggested that the VBP-decorated nanoparticles would be capable of binding to vWF without interfering with natural platelet's binding to the same vWF. Liposomes decorated with only CBP moieties exhibited significant binding to collagen-coated surfaces under flow, at all shear conditions. With a vision for mimicking platelet's synergistic adhesion mechanisms, the VBP and CBP moieties were co-conjugated on liposome surface, and the resultant heteromultivalently decorated particles showed significantly higher adhesion to 'vWF + collagen' surfaces at low-to-high shear ranges, compared to liposomes bearing VBP or CBP moieties alone. Interestingly, when compared to liposomes surface-decorated heteromultivalently with rGPIbα and CBP, liposomes surface-decorated with VBP and CBP showed significantly higher levels of adhesion on 'vWF + collagen' surface, suggesting that the large rGPIbα fragments may cause steric masking of smaller CBP moieties that can be avoided by using the VBP moieties.[199] This emphasizes the utilization of small peptides when heteromultivalent functionalization is needed for synergistic bioactivity, e.g. in nanoparticle-based mimicry of the dual adhesion mechanisms of platelets.

C.2. Nanomedicine systems inspired by platelet aggregation mechanisms

For the hemostatic action of natural platelets, adhesion to vWF and collagen at the injury site triggers several signal transduction pathways that ultimately lead to platelet activation, resulting in change in platelet morphology from biconvex discoid to stellar and spreading of platelets at the injury site.[201,202] These activation mechanisms also result in stimulation of platelet surface integrin GPIIb-IIIa from a resting to a ligand-binding conformation, that can bind the blood protein fibrinogen (Fg) via peptides RGD and HHLGGAKQAGDV (also known as H-12) in the α and γ chains on both termini of Fg.[203,204] As a result, active platelets can undergo Fg-mediated aggregation at the vascular injury site. The activated platelets also secrete agonists like adenosine di-phosphate (ADP), as well as facilitate generation of thrombin, that can further activate neighboring platelets, adding to the aggregating population. Rationalizing from these processes, a large volume of research has been carried out to mimic platelet's aggregation mechanisms by decorating synthetic particle surfaces by Fg itself, Fg fragments or Fg-based RGD and H-12 peptides. Some of the earliest designs of this approach involved surface-engineering of polymeric beads and RBCs with Fg or Fg-derived RGD peptides.[205-209] These designs were essentially 'super-fibrinogen' systems where the particle (or RBC) surface-decoration with Fg or Fg-derived

RGD peptides were meant to amplify the aggregation of active platelets via multivalent interaction with the stimulated GPIIb-IIIa integrins on activated platelet surface. These designs demonstrated hemostatic promise in vivo in several pre-clinical models, but have failed to progress into clinical translation, possibly due to the limitations of obtaining sufficient antigen-matched autologous RBCs (or from universal donors). To address such limitations, some research efforts have been directed toward creating 'universal donor-like RBCs' by masking RBC surface antigens with synthetic polymer coatings, or enzymatically converting the A and B antigens into O type, or stimulating stem cells into becoming universal donor type RBCs.[210] The scale-up efficacy and translational promise of such approaches are yet to be determined. Another Fg-based platelet-relevant nanotechnology approach that has undergone preclinical and clinical evaluation in the US and Europe is Fibrocaps™ (ProFibrix, The Netherlands), where a solution of fibrinogen and thrombin are separately spray-dried and then combined to produce a mixture of suspendable microparticles.[211] Fibrocaps have been used in clinical trials for treatment of bleeding during surgery and trauma-related injury, and have shown significant reduction in time-to-hemostasis after direct administration to surgical wound within a Gelatin Sponge, compared to the sponge alone. One limitation of this product is that it needs to be directly applied to the wound (sprayed or applied in a sponge) and therefore can only be applied to accessible wound sites and not intravenously. Several other Fg-based products that have undergone extensive research as platelet-inspired semi-synthetic hemostats are Synthocytes™, Thrombospheres™ and Fibrinoplate™, all of which are essentially made of human albumin microparticles surface-coated with Fg.[212,213] These products have undergone preclinical in vivo studies and some early phase clinical trials, but more detailed clinical evaluations are needed to establish their in vivo safety profile and treatment efficacies. In an analogous approach, Fg has also been coated on the surface of liposomes and the resultant vesicles have shown the ability to increase platelet recruitment and aggregation on collagen-covered surfaces in vitro. Another interesting nanomaterial reported in recent years is fibrin-targeted microgel and nanogel systems that can respond to hemodynamic environment at an injury site to potentially enhance platelet recruitment and aggregation, as well as, directly seal the bleeding site. This research has been demonstrated recently with polyispopropyl acrylamide based low-crosslinked mircogel particles surface-decorated with fibrin-specific recognition motifs, that can enable binding to injury site by specific interaction with nascent fibrin protofibrils.[214] Mechanistically, the binding of these constructs would require prior presence of sufficient fibrin (i.e. sufficient propagation of coagulation mechanisms) at the injury site. Therefore, rationalizing from the reported use of fibrin-specific constructs for targeted drug delivery to thrombi, these gel particles may find effective applications in clot-targeted therapeutic delivery.[215]

Using Fg to surface-coat micro- and nanoparticles for mimicking and leveraging platelet's hemostasis (and thrombosis)-relevant aggregation mechanisms may present several issues of storage stability, immunogenicity and off-target activity. A refined approach utilizing the same design rationale is to use Fg-relevant small molecular weight peptides for particle surface-decoration. The earliest examples of this are found in designs utilizing RBCs or polymeric particles with Fg-relevant RGD peptides, and similar approaches have also been reported in recent studies.[205,216] Most of these approaches have utilized linear small RGD

peptides, e.g. CGRGD or GRGDS, that have binding capability to platelet surface integrin GPIIb-IIIa. Two potential issues with using these peptides are (i) their ubiquitous nature to bind to many different integrins on other cells (i.e. lack of platelet-specificity) and (ii) their reported ability to trigger activation of resting platelets (i.e. systemic pro-thrombotic risk).[217,218] Nonetheless, from a feasibility demonstration standpoint, decoration of micro- and nanoparticles with these peptides have resulted in platelet-mimetic constructs that have shown promising hemostatic ability in vitro and in vivo.[216,219] The issue of specificity can be potentially resolved by utilizing peptides that have higher selectivity to the active (Fg-binding) form of platelet GPIIb-IIIa. To this end, researchers have used Fg γ-chain relevant H-12 peptide (i.e. HHLGGAKQAGDV) or Fg function-mimicking GPIIb-IIIa-specific cyclic RGD peptides (e.g. cyclo-CNPRGDY[-OEt]RC) to decorate albumin, polymeric or liposomal particles to create synthetic platelet mimics, that may provide higher functional specificity in hemostatic action.[220-224] The H-12 peptide decorated liposomal particles have been further studied for targeted delivery of ADP (a platelet agonist) to augment hemostatic capability and the H-12 peptide has also been used to surface-decorate disc-shaped 'nanosheets' made of poly-lactate-co-glycolate (PLGA) polymer, for potential topical hemostatic applications.[225,226] All of these micro- and nanoparticle designs that utilize peptides to mimic Fg-mediated platelet aggregation mechanisms, have shown the capability of inducing platelet aggregation *in vitro*, and some have shown hemostatic promise *in vivo* in a variety of pre-clinical animal models including tail vein bleeding, tail transection, femoral artery bleeding, liver resection and blunt trauma. Clinical translation of these designs and technologies will be dependent on establishing large scale manufacturing processes, demonstrating sterilizability and long shelf-life without compromising bioactivity, and evaluating systemic safety, circulation lifetime and hemostatic efficacy in larger animals of chronic and acute bleeding scenarios.

C.3. Nanomedicine systems that combine platelet's adhesion and aggregation mechanisms

Platelet-mediated hemostasis is rendered by the cooperative action of the adhesive and aggregatory functionalities of platelets.[227,228] Therefore, in recent years there has been significant interest in developing nanomedicine systems that can combine these two functions on a single particle platform. To this end, latex beads were surface-decorated simultaneously with rGPIbα protein fragments and H-12 peptides and liposomes were surface-decorated with rGPIbα protein fragments and collagen-binding peptides.[199,229] Studies with these constructs revealed that while combining two different motifs on a nanoparticle surface (i.e. heteromultivalent modification), if there is a significant size difference between the motifs (e.g. large rGPIbα fragment compared to small peptides), the motifs need to be presented at different canopy levels from the particle surface by using different spacer lengths, in order to minimize steric interference (or masking) of the smaller motifs by the larger motifs. Such steric interference issues can be potentially resolved by using only small peptides to combine the adhesive and aggregatory functions of platelets. This was demonstrated recently by combining the platelet-mimetic adhesion-promoting peptides VBP and CBP with the platelet-relevant aggregation-promoting cyclic RGD-based Fg-mimetic peptide (FMP) on liposomes or albumin-based particles.[224,230-232] These 'functionally integrated' platelet-mimetic systems showed higher hemostatic efficacy in vitro

(in flow chamber set-up), as well as, in vivo (in mouse tail transection bleeding model), compared to systems that had adhesion functionality only or aggregation functionality only. The liposome-based design was recently issued a patent (US 9107845) and registered as 'SynthoPlate^{TM}' and is being tested for treating thrombocytopenia as well as trauma, in pre-clinical animal models. These studies suggest that mimicking platelet-relevant multiple hemostatic functions via heteromultivalent ligand modifications on biocompatible nanoparticle platforms may lead to superior designs for synthetic platelet substitutes.

Synthetic Blood Substitutes

Integration of biomaterials engineering, blood biology and transfusion medicine areas has led to development of bio-inspired technologies that simulate the functions of RBCs, platelets, WBCs and plasma. For synthetic RBC substitutes, the most studied designs are hemoglobin (Hb)-based oxygen carriers (HBOCs) where hemoglobin molecules are directly polymerized, crosslinked, covalently modified with stability-enhancing polymers or encapsulated within micro- or nanoparticles. Since the O_2-transporting mechanisms of hemoglobin require enzyme-mediated redox environment, newer HBOC designs have co-encapsulated suitable enzymes and redox agents with Hb. Other promising approaches for oxygen carriers include perfluorocarbon (PFC) emulsions and synthetic porphyrin systems. For synthetic platelet substitutes, the most studied designs are essentially 'super-fibrinogen' systems where micro- or nanoparticles are surface-coated with fibrinogen, fibrinogen-relevant peptides or fibrin-specific motifs to amplify the fibrinogen-mediated 'aggregation' mechanism of active platelets. Some approaches have also focused on mimicking the 'adhesion' mechanisms of platelets by modifying nanoparticle surfaces with recombinant GPIbα or peptides to bind von Willebrand Factor (vWF) and recombinant GPIa-IIa to bind collagen. Newer designs have focused on combining the platelet-inspired 'aggregation' and 'adhesion' mechanisms via heteromultivalent modification of nanoparticles with vWF-binding, collagen-binding and GPIIb-IIIa-binding peptides. Other interesting approaches include surface-decoration of nanoparticles with polyphosphate (PolyP) moieties to modulate coagulation mechanisms and biointerfacing of polymeric nanoparticles with blood cell-derived membrane to impart specific bioactivities. Recent studies have also focused on incorporating RBC and platelet-inspired shape, size and mechanical flexibility aspects in the design of synthetic RBCs and platelets. As for plasma substitutes, several crystalloid and colloid compounds are currently clinically approved.

D. Synthetic nanomedicine systems using RBC and platelet membrane-based 'cloaking'

A persistent challenge in nanomedicine design is the interaction of nanoparticles with immune cells in vivo, resulting in rapid uptake and clearance of the particles by phagocytic processes of the mononuclear phagocytic system (MPS). One way to reduce such clearance is the decoration of nanoparticle surfaces with hydrophilic polymer brushes (e.g. with polyethylene glycol or PEG) that reduces opsonization of particles in blood circulation and thereby reduces phagocytic recognition and uptake. This approach has come to be known as

the ‘Stealth Nanoparticle’ technology.[88,89,233] In recent years, researchers have focused on another way to reduce this clearance by utilizing particle surface presentation with ‘markers of self’ to evade immune recognition. To this end, a ‘marker of self’ biomolecule that has garnered tremendous interest is CD47, an integrin-associated cell surface glycoprotein reportedly found on RBCs and polymorphonuclear leukocytes.[234] Surface modification of nanoparticle systems by CD47 moieties has demonstrated a reduction in macrophagic uptake and clearance.[235] Rationalizing from this immune-evading promise of CD47 moieties and based on the fact that RBCs bear these moieties, researchers have demonstrated an interesting approach of cloaking synthetic nanoparticles (e.g. PLGA nanoparticles) with isolated RBC membranes to create biosynthetic nanomedicine systems for drug delivery.[236-239] Such RBC membrane based bio-interfacing of nanoparticles has demonstrated notable increase in circulation lifetime of the particles, as well as, ability of the particles to allow passive targeting-based drug delivery to tumors.[240] In analogous approaches, various research groups have also reported the development nanoparticles coated with isolated platelet membranes, and these biosynthetic particle systems have shown the ability of delivering drugs to platelet-interactive disease states like metastatic tumors and bacterial infections.[241-243] While bio-interfacing of nanoparticles with isolated RBC and platelet-derived membranes represent an interesting approach to leverage biological complexity of native cell-surface moieties on synthetic particle platforms, this may also also present significant challenges regarding clinical translation. For example, utilization of RBC-derived membranes would necessitate either type matching of RBCs or universal donor (type O) RBCs or utilization of patient’s own RBCs to create patient-specific systems to minimize immunogenic risks. Similarly, utilization of platelet-derived systems would necessitate ensuring minimal immunogenicity and batch-to-batch reproducibility of membrane surface-protein composition to guarantee clinically translatable consistent bioactivity of the resultant nanoparticle technologies.[244] It has also been reported that extraction, isolation and purification processes applied to cell membrane systems often result in significant loss of membrane protein functions and high heterogeneity in residual bioactivities.[245] This can become a significant logistical barrier for quality control and functional uniformity of these membrane-derived bio-interfaced nanomedicine systems compared to fully synthetic (e.g. function-specific synthetic peptide decorated) nanomedicine systems.

E. Synthetic nanomedicine strategies for WBC substitutes

The primary function of circulating WBCs is to maintain immune surveillance and protection against foreign entities (e.g. bacteria) and diseased cells, and render cytotoxic destruction and phagocytic clearance of these entities via complex concert of signal transductions, intercellular interactions and cellular secretions. These complex multifactorial processes have not been leveraged and mimicked effectively on synthetic micro- or nanoparticle platforms. However, there has been some interesting synthetic approaches regarding functional mimicry of WBCs, e.g. (i) engineering of ‘Leukosomes’ where membrane glycoproteins from murine macrophages were extracted and reconstituted into proteoliposomes that could deliver drugs (e.g. Dexamethasone) to inflamed vasculature, (ii) engineering of ‘Leuko-polymerosomes’ where polymeric vesicles were heteromultivalently

surface-decorated with selectin- and integrin-binding motifs to render leukocyte-mimetic interactions with 'diseased' cells for targeted release of hydrogen peroxide (H_2O_2) from these vesicles to kill the target cells, and (iii) engineering of 'Leuko-like Vectors (LLVs)' where nanoporous silica particles were surface-coated with cellular membranes isolated from freshly harvested leukocytes to render properties like avoidance of opsonization and macrophagic uptake, preferential binding to inflamed endothelium, and facilitated transport of therapeutics across the endothelium while avoiding the lysosomal entrapment.[246-248] Figure 9 shows schematic representation of these various nanomedicine design approaches based on leukocyte biology. Further rigorous in vitro and in vivo evaluation of each of these approaches are needed to ensure manufacturing reproducibility, consistent composition and bioactivity of the surface motifs, in vivo safety and therapeutic promise.

F. Nanomedicine systems mimicking physico-mechanical properties of blood cells

The morphology and physico-mechanical properties of blood cells, especially RBCs and platelets, play important role in their collisional interactions under hemodynamic flow environment and thereby influence their margination in flowing blood.[159,249-253] RBCs are biconcave, discoid, highly flexible cells of 8-10 μm in diameter, while circulating 'resting' platelets are biconvex, discoid, relatively stiffer cells, about 2-3 μm in diameter. Under hemodynamic flow environment, these physico-mechanical properties influence RBCs to congregate at the center of blood vessels while platelets are pushed out to marginate towards the vessel wall in a relatively cell-free zone.[157,158] Based on these observations, some research approaches have tried to mimic the shape, size and flexibility of RBCs or platelets in nanomedicine design. Developing processes for the manufacture of non-spherical, anisotrpic particles has become an exciting area of nanomedicine research in recent years, because the resultant particles show intriguing aspects regarding their hemodynamic localization, circulation life-time and interactions with target cells and tissues.[254] Some of these processes have been utilized to manufacture micro- and nanoparticle systems that mimic shape, size and flexibility aspects of RBCs and platelets. For example, using a process called particle replication in non-wetting templates (PRINT®), researchers have manufactured RBC-mimetic discoid microparticles that showed higher circulation lifetime in vitro and in vivo.[119,255,256] RBC-mimetic and platelet-mimetic particles have also been developed utilizing techniques that use thermo-stretching of polymer templates followed by layer-by-layer (LBL) assembly of polyelectrolytes on templates and selective dissolution of templates, and these have shown significant promise regarding in vivo biodistribution, microfluidic margination, wall adhesion and drug delivery.[231,257-259] In a recent report, compared to stiff spherical particles, platelet-mimetic softer discoid particles exhibited better margination and adhesion (lower k_{off}) as measured by the duration of particle contact with the surface.[260,261] In further advancement of this research, platelet-mimetic soft discoid particles were surface-decorated heteromultivalently with VBP, CBP and RGD peptides, and these functionally integrated 'platelet-like nanoparticles' (PLNs) exhibited enhanced hemostatic efficacies in vitro and in vivo, compared to stiff spherical particles.[231] Studies have also been carried out with rGPIbα-decorated liposomes having various lipid membrane fluidities that result in various levels of membrane flexibility.[194] In these studies it was

found that “soft” liposomes rolled slowly and showed better adhesion on vWF-coated surfaces, compared to “hard” liposomes. Figure 10 shows a couple representative schematics of anisotropic discoid particle fabrication technologies and corresponding fluorescence or SEM images of the particles. These studies indicate the promise of incorporating RBC- or platelet-inspired biophysical design parameters to further refine the design of bio-inspired drug delivery particles.

Heteromultivalent Modification and Integration of Biochemical and Biophysical Parameters

In many biological mechanisms, cell-cell interactions and cell-matrix interactions are mediated by a concert of heterotypic ligand-receptor mechanisms rather than a single type of ligand-receptor interactions. In case of blood cells, this is found extensively in the intercellular as well as cell-matrix interactions of platelets and WBCs. Recent advancement in nanomedicine research has focused on elucidating and exploiting these heteromultivalent interactions in enhancing the bioactive performance of nanoparticle systems. For example, WBC interactions with inflamed endothelium are mediated by a combination of integrin-based and selectin-based binding and rolling mechanisms. Platelet interactions at the injury site are mediated by a combination of vWF-binding, collagen-binding, integrin-binding and selectin-binding mechanisms. Such combinations can be efficiently simulated on nanoparticle platforms by controlled co-decoration of the particle surface with multiple types of ligands. In carrying out such heteromultivalent decorations, researchers should ensure that the decorated motifs do not sterically interfere with each other and this can be achieved by controlling the size as well as the spacer lengths of motif conjugation. Another emerging area of cell-inspired nanomedicine research is the utilization of cell-mimetic biophysical parameters, e.g. size, shape, mechanical modulus, surface charge etc., combined with the cell-inspired biochemical (e.g. ligand-receptor interactions) parameters. Bottom up fabrication techniques like self-assembly and emulsion-directed precipitation, as well as, top down fabrication techniques like controlled lithography, hydrodynamic jetting, non-wetting templates, thermostretching etc. can provide effective ways to incorporate such morphological and physico-mechanical parameters in design refinement of cell-inspired nanomedicine systems.

G. Conclusion

Blood substitutes and artificial blood components remain highly sought after technologies that can potentially resolve several limitations associated with the widespread use of natural blood products, e.g. limited supply, expensive isolation and storage, limited portability, pathologic contamination risks and biological side-effects. The use of milk, saline, Ringer’s solution or animal-derived plasma to substitute for human blood, have been recorded in history during the 19th century.[262] These materials could partly act as volume replacement systems to maintain osmotic balance (and possibly provide nutrition), but did not have any component that essentially simulates the function of the various blood cells. Subsequent scientific research in this area of volume replacement therapies has led to the development of

plasma expander technologies under two main classes of materials, namely, crystalloids (e.g. sucrose, dextrose etc.) and colloids (e.g. albumin, hydroxyethyl starch etc.).[263,264] While the identification of blood groups and their significance in guiding blood transfusion happened during the late 1800s and early 1900s, the research is synthetic 'functional' substitutes of blood cells started only around the 1960s with the discovery of perfluorocarbons and this research intensified from the 1980s onward because of blood transfusion-related HIV risk issues. Since then, nanotechnology, materials engineering and deeper understanding of blood cell biology have provided unique avenues to design semi-synthetic and synthetic systems to mimic functions of RBCs, platelets and leukocytes. To this end, bio-inspired approaches for mimicking RBC function have focused mostly on capturing the oxygen transporting properties of Hb, either by using cross-linked and polymerized Hb directly or encapsulating Hb within a variety of microparticle and nanoparticle systems. The persistent challenges in these approaches are (i) limited availability of obtaining Hb from human and bovine sources and infection risks associated with these sources, (ii) limited mutation combinations and high cost of obtaining Hb from recombinant sources, (iii) maintaining consistent high loading efficiency of Hb, (iv) presenting of the redox environment that modulates Hb-mediated O_2 and CO_2 transport, (v) maintenance of reasonably long circulation lifetime, (vi) degradation or leakage of Hb within circulation and (vi) minimization of NO-scavenging by Hb leading to a variety of vascular and toxic side effects. Although some of the Hb-based synthetic RBC designs have partly addressed these challenges, none has been able to completely resolve these issues yet and that has remained as the barrier to clinical translation. Newer RBC-inspired nanomedicine designs like Hb intermolecularly and intramolecularly crosslinked with pharmacologically active agents, Hb polymerized with effector molecules and anti-oxidant enzymes, Hb encapsulated along with redox enzymes and effector molecules within liposomes, polymersomes or polymer-based toroidal nanoparticles, may provide refined strategies to resolve these issues. PFC-based microparticle and nanoparticle systems have already undergone clinical approval for contrast agent applications in ultrasound imaging and therefore leveraging this technology to clinically translate Hb-independent synthetic oxygen carriers, may be another promising option. In the field of bio-inspired approaches for mimicking platelet function, majority of research has focused on capturing the aggregation mechanism of active platelets by coating synthetic microparticle and nanoparticle platforms with Fg, Fg-fragments, Fg-relevant peptides or fibrin-binding motifs. While many of these designs have shown in vitro and in vivo hemostatic capabilities, they may also present systemic pro-thrombotic and pro-coagulant risks. Recent refinement of this design approach has focused on incorporating and combining platelet-mimetic injury site-adhesive functions along with active platelet aggregatory functions and these nanomedicine designs have shown significant improvement in promoting hemostasis in a targeted fashion while minimizing systemic off-target activity. An important aspect of this design refinement is to ensure that the particle surface-decorating peptide ligands have specificity of interactions with active platelets and injury site-relevant exposed proteins (e.g. vWF, collagen), so that they do not interact with circulating resting platelets to cause systemic thrombotic risks or promote thrombin and fibrin generation in plasma to cause systemic coagulation risks. Another interesting research possibility in this area is the utilization of nanoparticle system for targeted delivery of coagulation-modulating agents like polyphosphates (PolyP), tranexamic acid (TXA) and

various recombinant coagulation factors.[265,266] Furthermore, an emerging area of research in the field of blood cell-mimetic nanomedicine is the recent exploration of morphological and physico-mechanical parameters (e.g. size, shape, stiffness, flexibility etc.) inspired by blood cells (e.g. RBCs and platelets) that can enhance the performance of synthetic microparticle and nanoparticle systems in the vascular compartment under hemodynamic flow environment. Integrating these biophysical aspects with the biochemical functional aspects of RBCs and platelets, can lead to superior technologies for synthetic blood substitute applications. As these technologies evolve, rigorous research should be performed to evaluate the biological performance of these technologies in standardized pre-clinical animal models, not only in the context of specific functions of the blood cells but also regarding in vivo safety, especially off-target side effects and immune response. Clinical translation of these bio-inspired nanomedicine systems for synthetic blood substitutes require a concerted effort from biomedical and materials engineers, blood biology experts, haematology and trauma clinicians, as well as, appropriate regulatory agencies, to establish standardized in vitro and in vivo evaluation metrics and suitable clinical studies. As recently highlighted in the ‘State of Science in Transfusion Medicine’ by the NIH, there is a significant clinical interest in donor-independent blood cells for transfusion applications.[267] While one arm of the research is currently focusing on generation of blood cells ex vivo, from precursor cells and stem cells using specialized bioreactors and cell culture technologies, a parallel arm is continuing to work on synthetic nanomedicine approaches for blood cell substitutes.[268-272] Robust research and rigorous pre-clinical and clinical evaluation of these technologies can lead to fully artificial blood substitutes for transfusion applications in the future.

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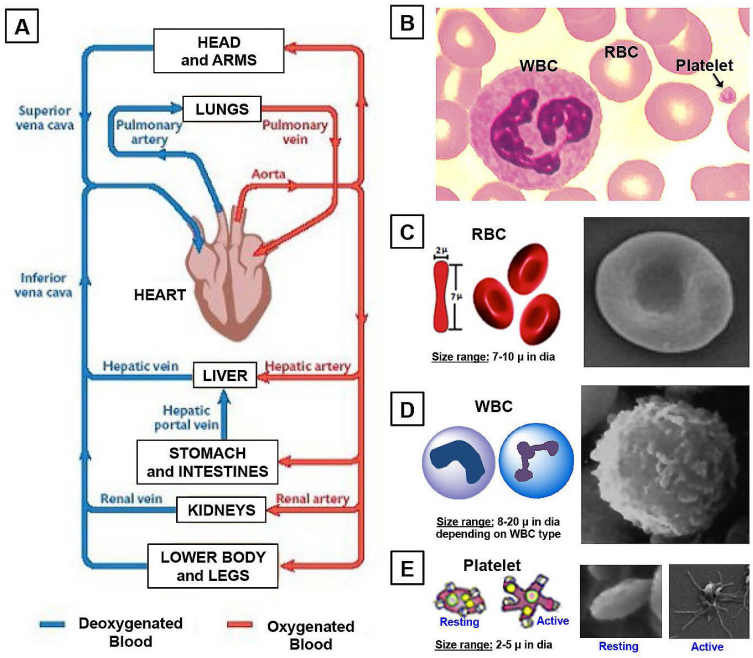


Figure 1.

[A] Arterial and venous circulation pathways in the body that carries RBCs, WBCs and platelets for respective biological functions; [B] Representative histology stain of blood smear showing RBC, WBC and platelet; [C] Schematic cartoon and representative SEM image of RBC; [D] Schematic cartoon and representative SEM image of WBC; [E] Schematic cartoon and representative SEM images of resting and active platelets.

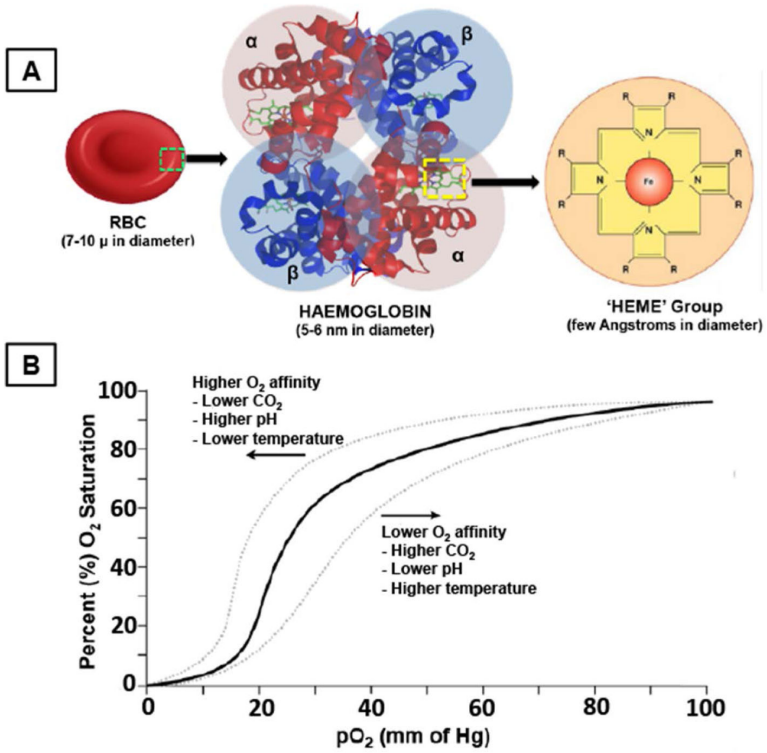


Figure 2.

[A] Multi-scale representation of RBC, the Hemoglobin (Hb) protein structure inside RBC and the 'Heme' porphyrin structure inside Hb; [B] Representative oxygen binding curve for Hb showing the co-operative binding nature (sigmoidal curve).

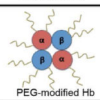
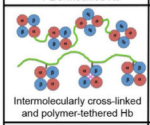
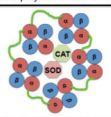
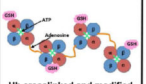
Chemically modified Hb based HBOC systems	Materials used	Representative Design Names
 Pure cell-free Hb	Cell-free Hb obtained from human, bovine, salmon and recombinant (e.g. in E. Coli) sources	Cell-free Hb
 Intra-molecularly crosslinked Hb	Cell-free Hb crosslinked between subunits with agents like glycine, glutaraldehyde, O-raffinose, 3,5-dibromosalicyl fumerate, pyridoxal-5-phosphate etc.	HemAssist (Baxter) Hemopure (Biopure) Optro (Somatogen) Hemolink (Hemosol) etc.
 PEG-modified Hb	Cell-free Hb conjugated on the surface by Maleimide-activated Polyethylene glycol (i.e. Hb-Mal-PEG)	Hemospan (Sangart) etc.
 Intermolecularly cross-linked and polymer-tethered Hb	Cell-free Hb intra- and inter-molecularly crosslinked or tethered from polymer chains by agents like glutaraldehyde, poly (oxyethylene), O-raffinose tec.	PolyHeme (Northfield Lab) Pyridoxylated Hb or PHP (Apex Bioscience) etc.
 Polymerized Hb incorporated with RBC-relevant redox enzyme systems	Cell-free Hb inter-molecularly crosslinked or polymer-tethered in multiple copies and associated with enzymes like superoxide dismutase (SOD), catalase (CAT), carbonic anhydrase (CA) etc. that can maintain the redox environment for efficient Hb activity	Poly-Hb-SOD-CAT, Poly-Hb-SOD-CAT-CA etc.
 Cell-free Hb surface-modified with albumin to form clusters	Cell-free Hb conjugated to human serum albumin (HSA) by reacting Hb surface lysines to HSA cysteine-34 via α-succinimidyl-ε-maleimide cross-linker, to form Hb-HAS nanoclusters; the HSA can further incorporate anti-oxidant enzymes and catalysts	Hb-HSA nanoclusters
 Hb crosslinked and modified with ATP, adenosine and GSH	Cell-free Hb cross-linked intra-molecularly with ATP, inter-molecularly with Adenosine and surface-modified with Glutathione (GSH) to provide pharmacologic and anti-oxidant activity	HemoTech (HemoBioTech)

Figure 3.

Schematic representation of design and components of RBC-inspired chemically modified prominent HBOC nanosystems, along with their design/trade names.

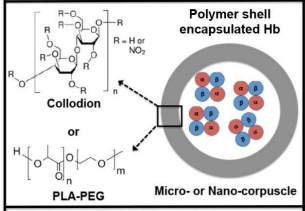
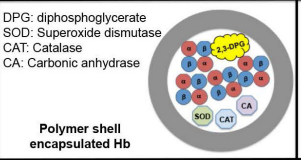
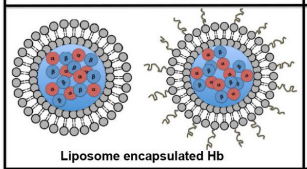
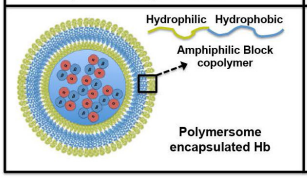
Encapsulated Hb based HBOCs	Materials used	Representative Design Names
	Hb encapsulated within Collodion (nitro-cellulose) membrane bound or PEG-PLA polymer membrane bound micro- or nanoparticles	Artificial Hb Corpuscle
	Hb encapsulated along with various redox enzymes within Collodion or PEG-PLA based membrane bound micro- or nanoparticles	Artificial Hb Corpuscle
	Hb encapsulated within sub-micron size lipid vesicles (liposomes) and PEG-ylated liposomes (i.e. 'Stealth' liposomes)	Liposome-encapsulated Hb (LEH), 'Neohemocyte', 'TRM-645 Neo Red Cells', Hb Vesicles (HbV) etc.
	Hb encapsulated within sub-micron size polymeric vesicles (Ipolymersomes) made from amphiphilic block copolymers like PEG-PBD, PEG-PLA, PEG-PCL etc.	Polymersome Encapsulated Hb (PEH)

Figure 4.

Schematic representation of design and components of RBC-inspired Hb-encapsulated prominent HBOC nanosystems, along with their design/trade names.

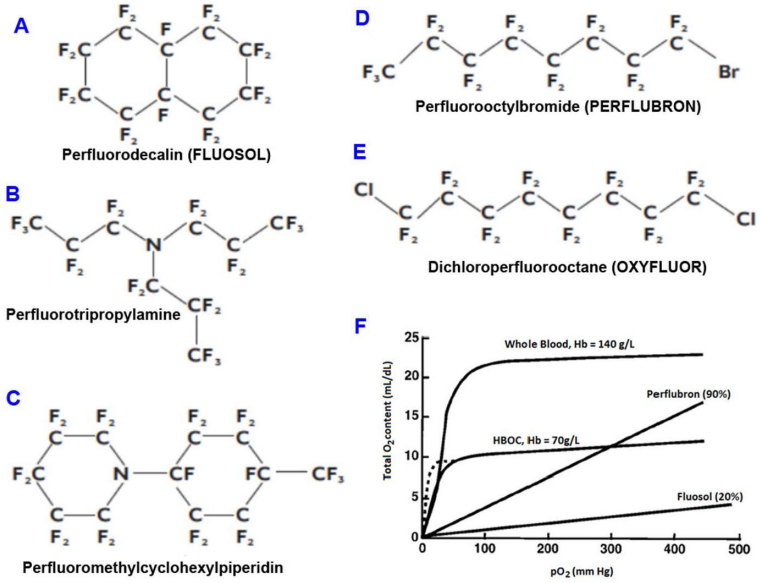


Figure 5.

[A]-[E] Chemical structure of various perfluorocarbon (PFC) compounds that have been studied for oxygen carrying applications; [F] Oxygen binding curves of whole blood and HBOC systems (cooperative sigmoid binding characteristics) compared with tat PFC-based oxygen carriers (linear binding characteristics).

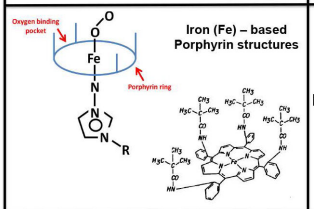
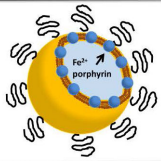
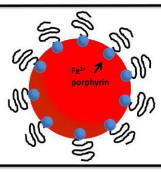
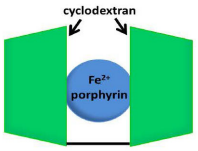
Synthetic Porphyrin based Oxygen Carriers	Materials used	Representative Design Names
	Ferrous 'heme' group embedded in a sterically hindered hydrophobic (e.g. polymer, albumin) matrix	'Picket Fence' Iron Porphyrin
	Amphiphilic Fe^{2+}-porphyrin bearing four alkylphosphocholine groups embedded into the bilayer membrane of phospholipid vesicles (Stealth liposomes)	LipidHeme
	Fe^{2+} porphyrin systems embedded within HSA microsphere structures	HSA-Heme
	Fe^{2+} porphyrin systems embedded inside the hydrophobic pockets of cyclodextran moieties	HemoCD

Figure 6.

Schematic representation of design and components of some porphyrin based oxygen-carrying nanosystems, along with their design/trade names.

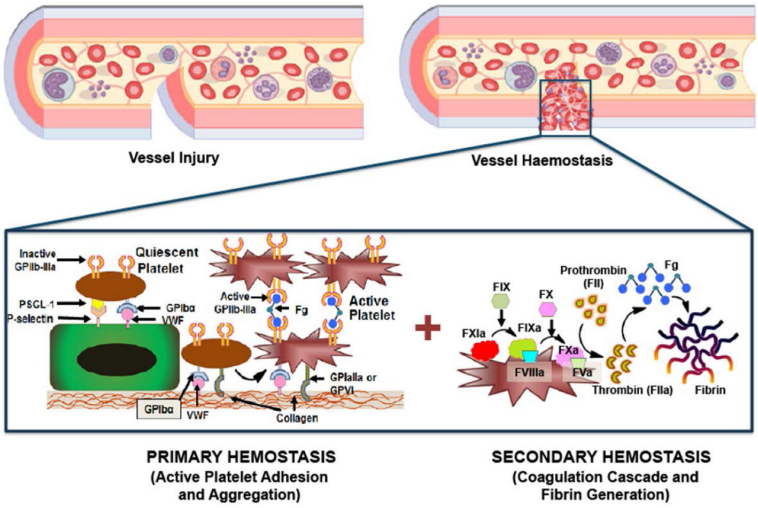


Figure 7.

Molecular mechanisms of platelet-mediated hemostasis in bleeding vessel, showing respective components of primary hemostasis and secondary hemostasis.

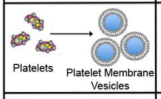
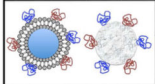
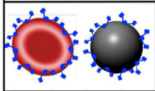
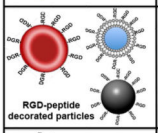
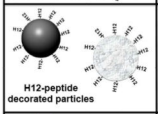
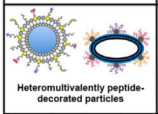
Platelet-inspired Nanomedicine Systems	Materials used	Representative Design Names
 Platelets Platelet Membrane Vesicles	Membranes from outdated platelets extracted by freeze-thaw, pasteurized and reconstituted into vesicles; in some cases, vesicle membrane further stabilized by trehalose	Infusible Platelet Membrane (IPM) Thrombosomes™ etc.
	Membrane glycoproteins extracted from natural platelets embedded in the bilayer membrane of liposomes, to conserve glycoprotein activity	Plateletsomes™
	Recombinant proteins mimicking platelet surface glycoproteins conjugated on liposomes and albumin nanoparticles to mimic platelet's adhesion mechanism	No specific product name reported
	RBCs, polymeric (e.g. acrylonitrile) beads or albumin microspheres surface-coated with fibrinogen (Fg) to mimic and amplifies platelet's Fg-mediated aggregation mechanism	Synthocytes™ Thrombospheres™ Fibrinoplate™ etc.
 RGD-peptide decorated particles	RBCs, Liposomes and polymeric (e.g. PEG-PLA) particles surface-decorated with CG**RGD** or G**RGD**S sequence of peptides that mimic the binding mechanism of Fg to platelet surface integrin GPIIb-IIIa	Thromboerythrocytes Synthetic Platelets etc.
 H12-peptide decorated particles	Polymeric (e.g. latex) beads and albumin microspheres surface-decorated with HHLGDAKQAGDV peptide sequence that mimics the binding of Fg to platelet surface integrin GPIIb-IIIa	No specific product name reported
 Heteromultivalently peptide-decorated particles	Liposomes and discoid albumin particles surface-decorated heteromultivalently with vWF-binding, collagen-binding and fibrinogen-mimetic peptides (VBP, CBP and FMP) to mimic and combine platelet's adhesion and aggregation functions	Platelet-like Nanoparticles (PLNs) SynthoPlate™ etc.
 Fibrin-binding microgel particles	Low crosslinked synthetic polymer (e.g. polyisopropyl acrylamide) microgel particles surface-decorated with fibrin-binding nanobody motifs	Platelet-like Particles (PLPs) etc.

Figure 8.

Schematic representation of design and components of platelet-inspired intravenously injectable synthetic hemostat nanosystems, along with their design/trade names where applicable.

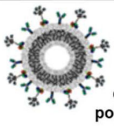
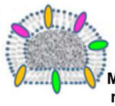
Leukocyte-inspired Nanomedicine	Materials used	Representative Design Names
 Macrophage membrane-based vesicles	Membrane of mouse macrophages extracted and reconstituted into proteoliposomes bearing various membrane components	Leukosomes
 Ligand-decorated polymersomes	Block co-polymer (e.g. PEO-b-PBD) based vesicles surface-decorated with Sialyl Lewis motifs and other cell adhesion molecules	Leukopolymersomes
 Macrophage membrane-coated particles	Porous silica nanoparticles coated with leukocyte-derived lipidic membranes bearing various membrane glycoprotein components	Leuko-like Vectors (LLV)

Figure 9.

Schematic representation of design and components of some WBC (leukocyte)-inspired biosynthetic nanosystems, along with their design/trade names.

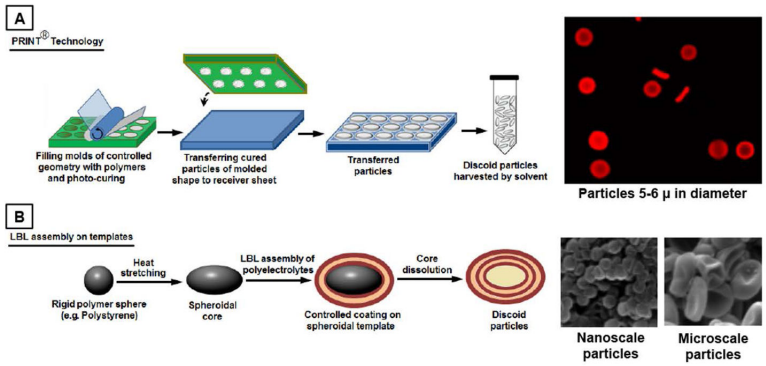


Figure 10.

Process scheme and representative particle images (fluorescence or SEM) for [A] the PRINT® technology and [B] the template-mediated thermostretching and layer-by-layer assembly based technology to produce micro- and nanoparticles that mimic the size and shape of blood cells (e.g. RBCs and platelets).