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Review article

Red blood cells-derived components as biomimetic functional materials: Matching versatile delivery strategies based on structure and function

Hangbing Liu [a,b,1], Yi Li [a,*,1], Yuli Wang [a,1], Liying Zhang [a,b], Xiaoqing Liang [a], Chunsheng Gao [a], Yang Yang [a,**]

[a] Beijing Institute of Pharmacology and Toxicology, 100850, Beijing, People's Republic of China
[b] School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, 110016, Shenyang, People's Republic of China

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ABSTRACT

Red blood cells (RBCs), often referred to as "intelligent delivery systems", can serve as biological or hybrid drug carriers due to their inherent advantages and characteristics. This innovative approach has the potential to enhance biocompatibility, pharmacokinetics, and provide targeting properties for drugs. By leveraging the unique structure and contents of RBCs, drug-loading pathways can be meticulously designed to align with these distinctive features. This review article primarily discusses the drug delivery strategies and their applications that are informed by the structural and functional properties of the main components of RBCs, including living RBCs, membranes, hollow RBCs, and hemoglobin. Overall, this review article would assist efforts to make better decisions on optimization and rational utilization of RBCs derivatives-based drug delivery strategies for the future direction in clinical translation.

1. Introduction

Drug delivery systems (DDS) are critical in improving the therapeutic efficacy of drugs within pharmaceutical research and development. These systems are designed to transport drugs to targeted sites in the body in a controlled manner, ensuring their release occurs at the appropriate time and location with pre-designed rates [1–4]. The core functions of drug delivery encompass targeting efficiencies, controlled release characteristics, facilitation of drug absorption, and enhancement of drug properties. Numerous researches have investigated a series of biomimetic micro/nano systems, such as various cells, viral vectors, and nanoparticles, via leveraging their unique functions for delivering drugs. However, existing issues have constrained their applications and clinical translation, such as the aggregation of polymer nanoparticles in the liver after administration [5], the high immunogenicity associated with lentiviral vectors [6], and the inefficiency and immaturity of exosome technology for preparation and storage [7]. The use of RBCs as drug carriers dates back over 70 years, with numerous pioneers making critical breakthroughs in this field. As early as the 1980s, it was demonstrated that RBCs could serve as targeted drug delivery carriers [8]. Various methods have been developed to load drugs onto the surface of RBCs, increasing the drug loading sites on these cells [9,10]. Additionally, RBCs have been combined with various nanocarriers, integrating the advantages of multiple carriers [11,12]. In vitro modifications of RBCs-DDS confer benefits such as enhanced targeting efficiency, prolonged circulation, reduced drug half-life, and controlled drug release [9,10,13,14]. RBCs and their derivatives including native RBCs, membranes of RBCs, hollow RBCs, and hemoglobin are regarded as one of the most promising drug carriers [15].

RBCs are the most abundant cells in the bloodstream. Healthy and living RBCs possess a biconcave disc shape (shown in Fig. 1), which provides the maximum surface area for the diffusion of gases (oxygen and carbon dioxide). The diameter of RBCs is approximately 6–8 μm, with a thickness of about 2 μm and a volume of approximately 90 fL [16]. Due to the absence of a nucleus and other organelles, RBCs can encapsulate a significant amount of contents like drugs or biomacromolecules [17], which on one hand safeguarding the encapsulated cargos from inactivation and degradation by endogenous factors, while

* Corresponding author.
** Corresponding author.
E-mail addresses: liu13342241@gmail.com (H. Liu), liyi_evening@pku.edu.cn (Y. Li), wangyuli764@126.com (Y. Wang), 3231854174@qq.com (L. Zhang), liangxq928@hotmail.com (X. Liang), gaocs@bmi.ac.cn (C. Gao), amms2013@126.com (Y. Yang).
[1] These authors contributed equally to this work.

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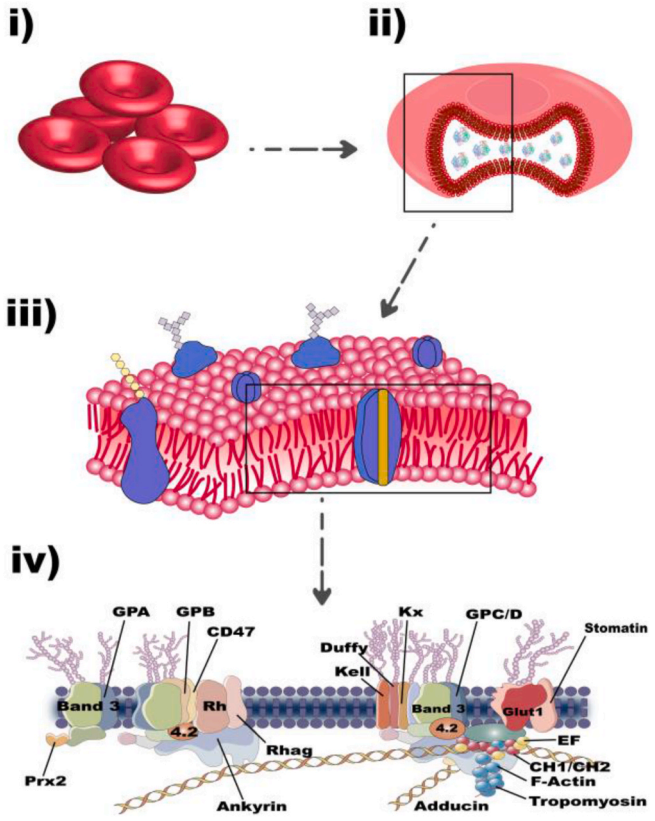

Fig. 1. Schematic diagrams of RBCM structures. i) Illustration of the biconcave disc shape of RBCs. ii) and iii) Composite structures of the lipid bilayer composed of amphipathic lipid molecules. iv) Various functional proteins anchored on the lipid bilayer of RBCM, which can be designed as the potential binding sites for drug coupling.

avoiding immune responses and toxic side effects of the encapsulated cargos on non-targeted organs on the other hand.

The lifespan of human RBCs is about 120 days and that of mouse RBCs is about 40 days. RBCs are naturally distributed throughout the body through the circulatory system, accessing various tissues, including hard-to-reach cancerous tissues, thus facilitating the specific distribution of the loaded drugs throughout the body. The loading of drugs/nanoparticles onto the surface or within RBCs can materially prolong the circulation time of drugs to fundamentally alter the pharmacokinetics (PK) and enhance the bioavailability of the drugs, thus leading to a lower Minimum Effective Dose (MED) and thereby mitigating the potential adverse reactions [18–20]. This approach also effectively deals with challenges related to the poor targeting and the premature clearance of nanoparticles [5,21,22].

Fully mature RBCs lose their ribosomes and consequently become incapable of synthesizing proteins [23]. The physiological and biochemical functions of aging RBCs undergo changes, including reduced metabolic activity, decreased cell volume, changes in cell morphology, and quantitative and qualitative changes in cell surface regulation [24]. The physiological changes in damaged RBCs are similar to those of aging RBCs. Aged or damaged RBCs are primarily cleared and recycled by the reticuloendothelial system (RES) in the liver, spleen, and bone marrow. Liver is the main organ for the clearance of aged and damaged RBCs, which are recognized and phagocytosed by the Kupffer cells (KCs) in liver [25]. While in spleen, aging and damaged RBCs enter the splenic sinus through narrow splenic cords and are captured by endothelial pores filled with monocytes and macrophages, as these cells cannot navigate within the splenic sinuses [26]. Based on the innate characteristics above, aged and damaged RBCs have been ingeniously repurposed as targeted delivery systems, specifically for the liver and spleen. This approach enhances the precision of drug delivery to these vital organs.

Leveraging the advantages of RBCs, their use as drug carriers holds tremendous potential in the field of drug delivery. RBCs can be loaded with drugs through various methods, including surface modification, internal encapsulation, and genetic engineering modification (Table 1), providing new avenues for precise, effective, and safe drug therapies.

Despite the significant potential demonstrated by intact RBCs delivery systems, their application is also faced with numerous limitations. For instance, these include low drug loading capacity, complex processing procedures, and potential risks of hemolysis. These shortcomings have prompted researchers to explore more flexible and efficient delivery systems, gradually shifting from the use of intact RBCs to designs based on their derived components.

2. Molecular structure of the red blood cells membrane (RBCM) and its application in drug delivery

Natural RBCM is primarily composed of lipids (accounting for approximately 44%), proteins (accounting for about 49% of the total weight), and also includes carbohydrates (Fig. 1).

2.1. Lipids

The lipids of the RBCM are primarily composed of phospholipids and cholesterol. Phospholipids account for 60% of the membrane lipids, while cholesterol and neutral fats make up 33% of the membrane lipids. The outer leaflet of the membrane is rich in phosphatidylcholine (PC) and sphingomyelin (SM, 23%), whereas the inner leaflet is mainly composed of phosphatidylethanolamine (PE), phosphatidylserine (PS), and a small amount of phosphatidylinositol (PI) (Fig. 3). SM and PC form the outer monolayer, while the majority of PE and all of PS form the inner monolayer, with a small amount of PI also present.

Lipid insertion methods can be applied in the preparation of functionalized RBCs with targeting, imaging, and therapeutic molecules [59–61]. Functionalizing RBCs carriers and RBCs-encapsulated nanoparticles through lipid insertion methods can prevent damage to RBCs caused by chemical reactions and facilitate the clearance of unbound lipid conjugates [62]. Small molecule folate-linked lipid conjugates physically inserted into the RBCM can produce functionalized RBCM for preparing RBC-NPs (Fig. 2A). The ligand-functionalized RBCM is used to coat polymer cores to form targeted RBC-NPs. Lipid insertion method does not require chemical coupling, which can protect surface proteins from damage by organic solvents and potentially minimize off-target side effects [62]. Li et al.'s research demonstrated that the Lys 248 site of human immunoglobulin (IgG) was selectively acetylated to obtain a monofunctional azide-IgG [63]. An antibody-lipid conjugate (ALC) was synthesized through azide-alkyne cycloaddition (SPAAC) with dibenzocyclooctyne (DBCO) functionalized lipids (Fig. 2B). The incorporation of the lipid component into the cell membrane was confirmed through laser confocal microscopy (Fig. 2C), thereby illustrating the successful construction of immuno-RBCs with IgG on their surfaces. These immuno-RBCs with the HER2-selective antibody trastuzumab displayed on the surface selectively target HER2-overexpressing tumor cells, and release drugs into cancer cells following photolysis, inducing photodynamic cytotoxicity in the cancer cells.

Different types of phospholipids play distinct roles within the cell membrane. The composition of lipids in the RBCM may vary under different conditions, with an uneven distribution of phospholipids on the membrane surface, which is related to functions such as blood coagulation, cell clearance, and membrane fusion [64].

2.1.1. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE)

PC is typically located on the outer leaflet of the cell membrane [65], composed of a choline "head" and a glycerol phosphate linked to two fatty acids. One of the tails is usually a saturated fatty acid (such as

Table 1
Main drug loading pathways for RBCs-based carriers.

Location	Method		Brief Description	Advantages	Disadvantages	Example	Ref
Surface	Coupling	Crosslinking agent	1) Cross-linking agents such as glutaraldehyde and ferric sulfate can be used to covalently link drugs to RBCM proteins. 2) Glycophorin A, a surface glycoprotein, served as a binding site for drugs. 3) PEG cross-linking agents can modify the RBCM to increase stability and biocompatibility.	Simple operation, high drug loading efficiency.	Osmotic stress during drug loading may damage the RBCM, leading to cytoskeletal dysfunction (loss of cell plasticity and mechanical stability).	RBCs composed of PEG cross-linking agent and protein A (SpA) covalently coupled. The Fc region of the antibody is shielded, allowing the antibody on the surface of RBCs to effectively clear antigens.	[27]
		Biotin−Avidin cross-linking	Streptavidin, an alkaline glycoprotein composed of four identical subunits, each of which provides independent binding site for a single biotin molecule.	The biotin-avidin system has strong specific binding capacity and high stability, unaffected by temperature, pH, and organic solvents.	Potential loss of activities and some functionalities of RBCs during the preparation process.	Biotinylated RBCs-poly (lactic-co-glycolic acid) nanoparticle hybrid DDS (bE-NP), which can mimic the characteristics of aging RBCs and encapsulate anticancer drugs.	[28–31]
	Affinity Interaction		Cargo is coupled to the RBCM through direct interaction of antibody-protein.	Bio-safety and high encapsulation efficiency. High specificity of antibodies and proteins in complex blood and vascular systems.	The antibody-protein structure is relatively small and can easily dissociate. Antibodies that occupy the inherent receptor recognition sites on the cell surface may shield these surface receptors, resulting in a shortened circulation half-life.	Tissue plasminogen activator (tPA) combined with monoclonal antibody 7G9, which is expressed on human RBCs, forms an anti-CR1/tPA conjugate. Rapidly alleviating mouse pulmonary embolism without affecting RBCs survival.	[32–35]
	Enzyme-Linked Reaction		Under the catalysis of protein ligases, such as OaAEP1 and Sortase A, proteins are covalently linked to the RBCM.	Simple and efficient, no chemical modification required, high biocompatibility, stable and not affected by acids and bases.	Ineffective in polymerizing multiple mixed forms of proteins, such as metalloproteases. Low catalytic efficiency, requiring a large amount of catalyst and longer reaction time.	Sortase enzyme covalently links autoantigen peptides to proteins on the RBCM. Prevention and treatment to experimental autoimmune encephalomyelitis (EAE) mouse model.	[36–38]
	RBCs-hitchhiking	RBCs-hitchhiking	Physically adsorb nanocarriers onto the surface of RBCs.	Significantly alter the PK profile of attached nanocarriers and significantly alters the biodistribution (BD) of nanocarriers.	Mechanisms of RBCs-adsorption and nanocarrier transfer are uncertain and relatively low efficiency of delivery.	RBCs-hitchhiking of NCs loaded with fibrinolytic plasminogen activators affords more effective dissolution of pulmonary thrombi in a mouse model of fibrin clots lodged in the lung vasculature	[39,40]
		Double-targeting RBCs-hitchhiking	The nano-carrier has dual affinity for erythrocytes and target cells.	The extension of half-life in circulation and reduction of RES uptake	DART may trigger an immune response in the host	DART nanocarriers accumulate in the target organ (lungs)。	[41]
Inside	Hypotonic Method		Formation of transient nanometer-sized pores on the RBCs surface.	1) High biocompatibility and extended half-life of drugs in the body. 2) The integrity of the RBCs structure ensures the stability of the internal drugs, avoiding drug decomposition. 3) Simple preparation method.	1) Inapplicability to larger nanocarriers; 2) Impact on the integrity of the red blood cell membrane and deformability.	Enzymes, lipids, and small molecule drugs are encapsulated inside RBCs. For example, autologous RBCs encapsulated with dexamethasone 21-phosphate (Dex 21-P) and kanamycin inside.	[42–44]
	Electroporation		RBCs are exposed to a high-voltage electric field. The increased permeability of the cell membrane allows drug molecules to enter RBCs from the extracellular fluid.	High drug encapsulation rate, high biocompatibility, reversible damage to RBCs' structure.	The electroporation technique is complex and difficult to implement. An exceeded electrical stimulus can directly lead to the rupture and death of RBCs.	This technique can be utilized for the encapsulation of small molecule drugs and enzymes, including alcohol dehydrogenase and aldehyde dehydrogenase.	[45–48]

(*continued on next page*)

Table 1 (*continued*)

Location	Method	Brief Description	Advantages	Disadvantages	Example	Ref
	Lipid Fusion	Fusing RBCs with drug-loaded liposomes to load drugs into the cytoplasm based on the principle of mutual solubility between phospholipids.	Surface-modified liposomes enhance the targeting of fusion RBCs to specific tissues or organs. Evasion of the immune system, achieving long-term circulation, significant inherent biocompatibility, and biodegradability.	Damage to the cytoskeleton and leakage of hemoglobin, thus affecting the function of RBCs in storing and transporting oxygen.	Liposomes loaded with doxorubicin (liposome) are fused with RBCM to form biomimetic liposomes (BML) for the treatment of osteosarcoma.	[49–51]
	Cell Penetrating Peptides (CCP)	Cargo such as proteins covalently bound with CCP allows internalization into cells through endocytosis without changing the structure or function of RBCs.	CCP has universal and effective membrane penetration activity, enabling the internalization of CCP-protein conjugates while maintaining the integrity of RBCs. Furthermore, CCP-linked large molecules can non- specifically traverse cell membranes of various tissues, including brain.	This method is not suitable for clinical application due to the potential risks of immune rejection and resistance.	Low molecular weight protamine (LMWP) is employed to mediate the encapsulation of L-asparaginase within intact RBCs for the treatment of acute lymphoblastic leukemia.	[52–54]
Cytoplasm and Surface	Genetic Engineering	Genetic modification or DNA recombination technology is used to modify hematopoietic stem cells or progenitor cells to express specific proteins or peptides in the cytoplasm or cell membrane of artificially induced mature RBCs.	The only method that can effectively engineer membrane transport proteins and other transmembrane proteins in a complete and functional state on the engineered cells (artificially induced mature RBCs) at present. Almost all proteins can be expressed on the cells.	1) Process for preparation is complex, and requires strict control over the expansion and differentiation of progenitor cells. 2) Low drug loading efficiency. 3) Lack of effective quality control measures. 4) Limited practical application.	RTX-240 is a genetically engineered RBCs that expresses 4-1BBL and IL-15/IL-15Rα fusion protein (IL-15TP), activating and expanding T cells and NK cells, thereby inhibiting tumor growth.	[55–58]

palmitic acid), while the other is an unsaturated fatty acid (for example, oleic acid). The head of phosphatidylcholine contains a choline group, which is involved in membrane-mediated cellular signaling and the activation of other enzymes. PE is the most abundant lipid in the RBCM, forming the bilayer structure of the biomembrane together with cholesterol and other phospholipids, providing stable support and protection for the cell. This structure not only helps the cell maintain its shape and integrity but also ensures the smooth exchange of substances and the transmission of information between the inside and outside of the cell.

2.1.2. Phosphatidylserine (PS)

PS is a prevalent anionic phospholipid found in eukaryotic cell membranes, constituting approximately 10% of the total lipid content within cells. The molecular structure of PS comprises a hydrophilic glycerol backbone serving as the head, along with two elongated hydrocarbon chains functioning as the hydrophobic tails. The enzyme P4-ATPase facilitates the translocation of phospholipids from the cytoplasmic leaflet to the exoplasmic leaflet of the cell membrane, thereby establishing and sustaining lipid asymmetry. The exposure of PS on the outer leaflet of the RBCM is a key signal for cell clearance signal for macrophages, and platelet coagulation [66,67]. RBCs' senescence is a form of spontaneous programmed cell death, distinct from the apoptosis of nucleated cells. The externalization of phosphatidylserine plays a crucial role in the process of RBCs' senescence (Fig. 3) [68]. On one hand, the externalization of PS is an important signal for macrophages in the liver and spleen to recognize and clear apoptotic cells [69]. PS receptors on the surface of macrophages recognize the externalized PS and initiate the phagocytic process, such as Tim1, Tim4 receptors, lactadherin, platelet response protein, Gas6, and protein S, which can promote macrophage recognition when interacting with exposed PS on RBCs [70–72]. On the other hand, various structures on the cell membrane surface affect the externalization of PS. Ion channels and pumps on the RBCM, such as the sodium-potassium pump, calcium pump channels, and pump dysfunction, may lead to changes in intracellular calcium ion concentration [73], thereby affecting the externalization of PS. Increased membrane fluidity may promote the externalization of PS, as PS molecules move more freely in a more loosely packed membrane. When the cholesterol content in the RBCM is reduced [74,75], the externalization of PS increases, which may make RBCs more easily recognized and cleared by phagocytic cells. For delivery systems using RBCs or their membrane structures as carriers, avoiding PS externalization can inhibit recognition and clearance by RES, thereby improving long-circulation characteristics; Conversely, altering various structures on the engineered drug-loaded RBCM to induce PS externalization can enhance its targeting efficiency to the liver and spleen.

2.2. Surface proteins of RBCM

RBCM contain a multitude of proteins, with over 50 membrane proteins that work in concert to maintain the physiological functions of RBCs (Table 2). These proteins either span the lipid bilayer once or multiple times or are anchored to the lipid bilayer through lipid tails [76]. Based on their functions, membrane proteins can be broadly categorized into three groups: structural proteins, physiological function proteins, and immunological recognition proteins.

2.2.1. RBCM structural proteins

The RBCM skeleton is a fundamental and complex structure formed by the cytoskeleton and the plasma membrane, primarily composed of a protein network located on the inner side of the cell membrane, providing critical support to maintain the biconcave disc shape of RBCs,

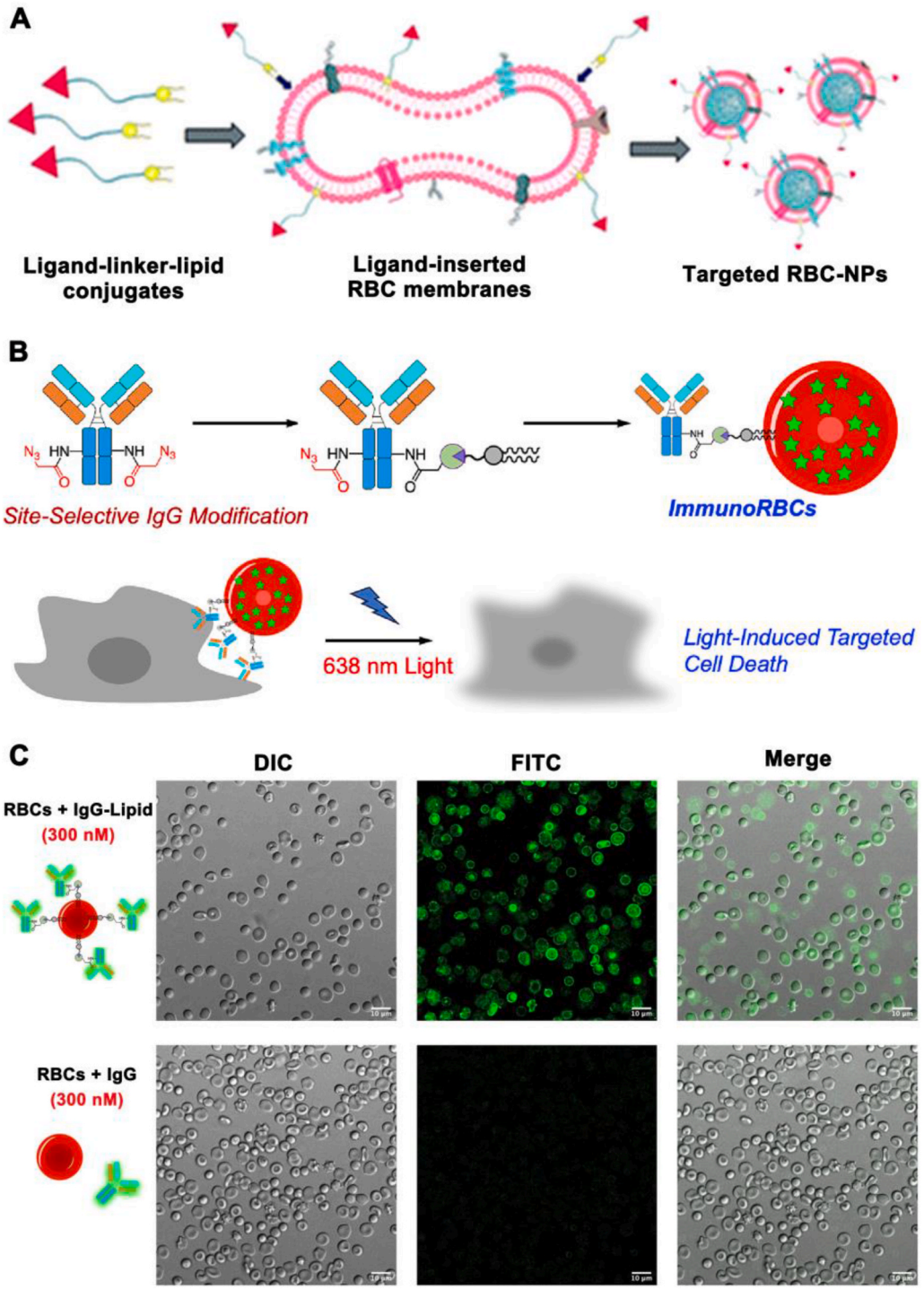

Fig. 2. Lipid insertion method for incorporating functional molecules into the RBCM. A) The targeting component is incorporated into the RBCM through a lipid tether. The physical insertion of ligand-linker-lipid conjugates into the RBCM can yield functionalized RBCM. Adapted with permission from Ref. [62]. Copyright © 2013 Royal Society of Chemistry. B) The modified IgG antibody-lipid conjugate (ALC) is inserted into the cell membrane to form immuno-RBCs, which release drugs into cancer cells to kill them. C) Fluorescent confocal microscope images confirm the decoration of the RBCs' surface with ALC, which aligns with the membrane insertion process. B) and C) are adapted from Ref. [63] with permission from Elsevier.

thus maximizing the surface area for oxygen exchange and also imparting elasticity and deformability to RBCs for facilitating their unobstructed passage through blood vessels. Additionally, the skeleton provides physical stability to prevent damage to RBCs during the turbulent blood flow. The primary components of the RBCM skeleton include ankyrin, Band 3 protein, and protein 4.1, which are interconnected at nodes to spectrin, thereby forming the intact structure.

2.2.1.1. Spectrin. Spectrin exists in two isoforms: the erythroid isoform (SpI), which is exclusively expressed in RBCs, and the non-erythroid isoform (SpII), found in platelets and neurons [77]. Spectrin is a filamentous protein composed of two subunits: 280 kDa alpha-spectrin consisting of 2419 amino acids and 246 kDa beta-spectrin consisting of 2137 amino acids [78]. Alpha-spectrin accounts for approximately 60% of the total spectrin and plays a crucial role in maintaining the

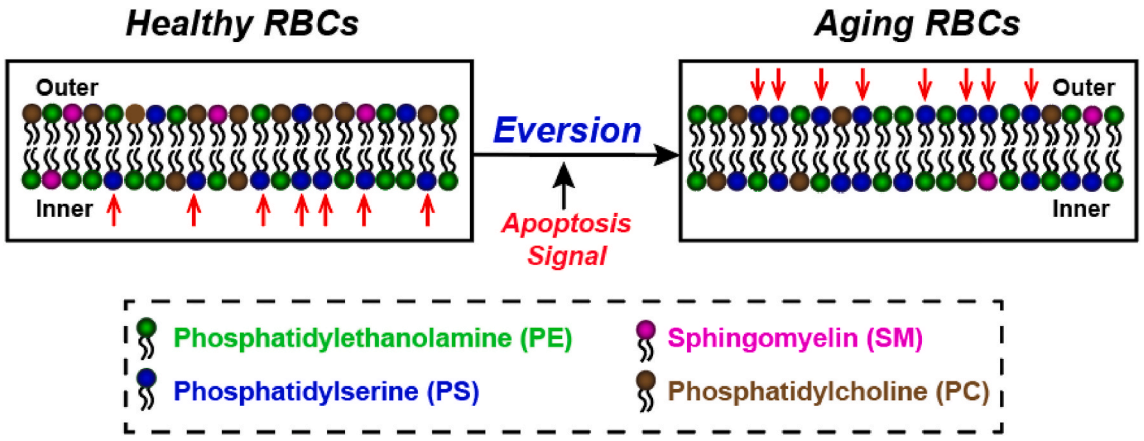

Fig. 3. Distribution of various phospholipids on the surface of RBCs.

elasticity and stability of RBCs. Beta-spectrin binds to alpha-spectrin to form the fundamental unit of the spectrin network. The two subunits combine head-to-head to form a dimer, which are interconnected at their tails to create a tetramer [79]. This tetramer connects to a junctional complex that comprises short actin filaments, ankyrin, adducin, protein 4.1, and related proteins [80]. The tetramers further assemble into a predominantly hexagonal polygonal network that underlies the inner side of the RBCM.

2.2.1.2. Band 3. Band 3 protein, also referred to as Anion Exchanger 1 (AE1), is primarily expressed in the membrane of RBCs, with lower expression observed on the basolateral side of the collecting ducts within the renal unit [81]. This 95 kDa protein is a multi-transmembrane protein that traverses the plasma membrane 12 to 14 times and constitutes approximately 25% of the total protein content in the RBCM [82].

The N-terminal cytoplasmic domain of Band 3 associates with intracellular proteins, such as ankyrin, which subsequently links to the spectrin tetramer within the cytoskeletal network, thereby preserving the biconcave shape of RBCs. Band 3 plays a critical role in transmembrane signaling and the regulation of various cellular functions, including cell growth, differentiation, and intercellular recognition. In RBCs, one of the physiological functions of the Band 3 protein is to facilitate the Cl^-/HCO_3^- exchange, which is essential for the effective transport of CO_2 in tissues and its expulsion from the lungs [83]. The Band 3 protein is encoded by the SLC4A1 gene, which participates in keeping the integrity of the membrane structure and withstanding deformation.

Monoclonal single-domain antibodies (sdAbs) specifically identifying Band 3 have been designed for the purpose of targeting Band 3, and they have undergone affinity maturation to enhance their binding capability with murine RBCs. Coupling these sdAbs to the Complement Receptor 1 (CR1), which is an anchor point for immune complexes containing activated complement C3b on RBCs, can significantly extend the in vivo half-life of small therapeutic proteins [84]. This approach leverages the natural mechanism of CR1 in regulating the immune response and clearing immune complexes from the circulation, thereby potentially improving the therapeutic efficacy and duration of action of the attached proteins.

RBCs can be more efficiently recognized by macrophages after being penetrated by enzymes, small molecules, and growth factors via hypotonic loading [85,86], similar to that of natural RBCs. Further, the effectiveness of macrophages to phagocytize RBCs that are crosslinked with drugs via Band 3 is significantly augmented. Following crosslinking with bis(sulfosuccinimidyl)suberate (BS_3) and 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP), the quantity of Band 3 protein on the RBCM is diminished by 17–21% [85]. Consequently, crosslinked carrier RBCs can be shown to facilitate liver targeting in vivo and to be recognized by macrophages, as demonstrated by chromium labeling analysis [87].

2.2.2. The application of RBCs' proteins with immune recognition function in drug delivery systems

RBCs possess a variety of proteins for immune recognition on the surface, such as CR1, Glycoprotein A (GPA), Intercellular Adhesion Molecule-4 (ICAM-4), CD59, Decay Accelerating Factor (DAF), and Glucose Transporter 1 (GLUT1), among others. These proteins serve as potential binding sites for therapeutic agents, allowing for the attachment of drugs to the RBCs' surface or the conjugation with targeting agents. Additionally, various membrane proteins contribute to mechanisms of immune evasion. By encapsulating biodegradable polymer nanoparticles with the natural RBCM, researchers have achieved prolonged circulation and effective drug delivery within the body, while simultaneously circumventing phagocytic uptake and systemic clearance [129].

2.2.2.1. CD47. CD47 is widely expressed in various cell types, such as RBCs, white blood cells, platelets, and vascular endothelial cells. CD47 is an integrin-associated transmembrane glycoprotein, primarily divided into three parts: i) extracellular N-terminal IgV domain that is responsible for interacting with other molecules such as SIRPα [130]; ii) transmembrane domain, characterized by five highly hydrophobic segments that ensure stable embedding within the cell membrane; iii) intracellular C-terminal domain, which is relatively shorter and can interact with specific ligands, mediating a range of biological processes, including cell proliferation, migration, phagocytosis, apoptosis, immune homeostasis, and the inhibition of nitric oxide signal transduction [113].

One of the primary functions of CD47 is to regulate the activation and phagocytic functions of immune cells through its interaction with SIRPα, which is located on the surface of immune cells. The binding of CD47 to SIRPα transmits a ''do not eat me'' signal, thereby preventing immune cells from attacking autologous cells [114,115]. CD47 greatly involves in extending the half-life of RBCs in systemic circulation, thereby improving the efficiency of RBCs-based drug delivery. RBCM-disguised polymeric nanoparticles were designed for evading phagocytosis and presenting reduced immune-related reactions through a rich amount of CD47 proteins expressed on the intact RBCM. Hu et al. demonstrated that RBCM-decorated biomimetic nanocarriers (RBC-NPs) with a particle size of 100 nm presented a surface density of CD47 similar to that of natural RBCs (Fig. 4A) [131]. Fig. 4B presents the proteomic analysis of poly(lactic-co-glycolic acid) (PLGA) nanoparticles enveloped by the RBCM. The lanes in Fig. 4B (left) represent proteins eluted from the nanoparticles, revealing the protein composition on the surface of the nanoparticles. Fig. 4B (right) displays a distinct single

Table 2
Structure and function of various membrane proteins on RBCs.

Protein	Gene	Structure	Function	Ref
spectrin (α and β)	SPTA1 and SPTB	Filamentous heterodimers, antiparallel arrangement forming tetramers approximately 200 nm in length.	Major component of the RBCM skeleton.	[79,88,89]
actin (Band 5 protein)	ACTB and ACTG1	Consists of 13 actin monomers and a long 35 nm tropomyosin molecule.	Actin filaments contribute to the membrane skeleton by binding to the free ends of spectrin.	[90]
glycophorin A (GPA)	GYPA	Sialoglycoprotein, single-pass transmembrane.	Prevents RBCs from aggregating and depositing in blood vessels during circulation.	[91,92]
Ankyrin	ANK1, ANK2, ANK3	Protein with a molecular weight of 215 kDa.	Ankyrin links the spectrin-actin network to the plasma membrane via Band 3 protein.	[93–95]
Band 4.1 protein	EPB41, EPB41L1, EPB41L3	Spherical protein composed of two subunits.	Facilitates the binding of spectrin to actin.	[96]
Adducin	ADD1, ADD2, ADD3	Dimer composed of two subunits.	Adducin binds to the spectrin-actin complex and affects stability through Ca^{2+} and calmodulin, influencing RBCs shape.	[97]
Band 3 protein (AE1)		Glycoprotein, dimeric structure of identical subunits.	Anion transport function.	[81,82]
PIEZO1	PIEZO1	Homotrimeric structure resembling a three-blade propeller with a central pore for ion permeation.	One of the largest ion channels in RBCs, plays a significant role in maintaining cell volume homeostasis.	[98,99]
KCNN4-Gardos Channel	KCNN4	Not fully determined three-dimensional structure.	Calcium-sensitive, medium-conductance, potassium-selective channel involved in cell volume regulation.	[100,101]
GLUT-1	SLC2A1	12 transmembrane helices forming two domains with an inward-facing conformation.	Responsible for constitutive or basal glucose uptake with broad substrate specificity.	[102–105]
Na^+-K^+-ATPase	ATP1A and ATP1B	α-subunit ~110 kDa, β-subunit ~50 kDa.	Maintains the gradient of Na^+ and K^+ across the RBCM.	[106,107]
RhAG	RhAG	Composed of 409 amino acids.	Membrane channel for transporting ammonium and carbon dioxide in RBCs.	[108]
LRRC8A	LRRC8A	Four-pass transmembrane protein forming heteromeric assemblies near the channel pore.	A key component of the volume-sensitive anion channel.	[109]
Aquaporin-1 (AQP-1)		A transmembrane protein consisting of four identical subunits, each with six transmembrane domains forming a highly selective water channel.	Key pore selectively for water transport, not allowing ions or small molecules to pass through.	[110,111]
G6PD	G6PD gene on X chromosome	A transmembrane enzyme composed of four identical subunits.	Catalyzes glucose-6-phosphate, producing NADPH which is a coenzyme for glutathione reductase for maintaining hemoglobin stability and RBCM integrity.	[112]
CD47	STAT3, β-catenin/TCF4, HIF-1, c-Myc	A transmembrane protein with an extracellular N-terminal IgV domain, five transmembrane domains, and a short C-terminal intracellular tail.	Inhibits phagocytosis by macrophages.	[113–115]
ST3GAL4	ST6Gal.I	A complex of protein and carbohydrates, with sugar chains primarily composed of galactose, fucose, N-acetylgalactosamine, and N-acetylglucosamine.	Involved in cell-cell interactions.	[116]
CD34 (ICAM-4)	microRNA hsa-miR-93	Protein containing 2 Ig-like C2-type domains.	Plays a key role in cell-cell interactions or adhesion.	[117,118]
CD36 (GP4)	CD36 gene on chromosome 7q11	Contains 2 transmembrane domains, a large extracellular domain with ligand-binding sites, and 2 short cytoplasmic tails at the N- and C-termini.	Absorbs long-chain fatty acids (LCFAs) and oxidized low-density lipoprotein (ox-LDL). Adhesion molecule for monocytes, platelets, and endothelial cells.	[119,120]
CD44 (HCAM)	CD44	A single-pass transmembrane protein composed of a single polypeptide chain.	Receptor for hyaluronic acid (HA), osteopontin, and fibronectin, mediating cell-cell matrix adhesion and participating in cell-cell communication.	[121]
CD35 (CR1)	Co-dominant genes L and H	Polymorphic single chain membrane binding protein	Complement receptor and has a variety of immune functions	[122]
CD55 (DAF)	PIG-A	Anchored to the cell membrane by glycosylphosphatidylinositol (GPI).	Protects RBCs from hemolysis mediated by the complement system.	[123–126]
CD59 (MACIF)	Gene on the short arm of chromosome 11 (11p13)	A protein anchored to the cell membrane surface by glycosylphosphatidylinositol (GPI). Contains 10 cysteines forming 5 disulfide bonds.	Complement regulatory protein.	[126,127]
Rh17	GATA1 and GATA2	Cell membrane surface proteins	It is related to the immunogenicity of RBCs and the compatibility of blood group system	[128]

band at the position of 50 kDa, which is the characteristic molecular weight of CD47 protein, confirming the successful transfer of CD47 protein from the RBCM to the surface of PLGA nanoparticles. CD47 was verified as the key molecule dominating the immune evasion of RBC-NPs from phagocytosis (Fig. 4C). Although polyethylene glycol (PEG) is widely used in nanoparticles for immune evasion to avoid clearance, the tendency of the immune system to eliminate heterologous substances still cannot be ignored. By substituting PEG with RBCM, it is showed that Fe_3O_4@RBC NPs were able to avoid immune clearance relying on the interaction of CD47 on RBCs with the SIRP-α receptor, exhibiting prolonged circulation time and reduced blood clearance rate [132]. Conversely, reducing or shielding CD47 on RBCs can increase the liver accumulation, with which features can be designed as drug carriers for liver targeting. Yao et al. reported a hydrogen peroxide-damaged RBCM as biomimetic material with significant lower level of CD47 than that of normal RBCM, wrapping nanostructured lipid carriers (NLC) loaded with the detoxifying agent dimercaptosuccinic acid (DMSA), significantly enhanced the liver-targeting efficiency of the delivery system for liver detoxification [133].

2.2.2.2. Complement regulatory proteins. CR1, also known as CD35, is a multifunctional protein on the surface of RBCs primarily involved in the regulation of the complement system. Its ligands include high-affinity C3b and C4b, as well as low-affinity C3bi and C3c [122]. Immune complexes containing the activating component C3b can bind to CR1 on RBCs, leading to their transfer to macrophages and subsequent clearance. CR1 is an early target for the binding of drugs to the RBCs' surface. Bispecific monoclonal antibody reagents connect the immune

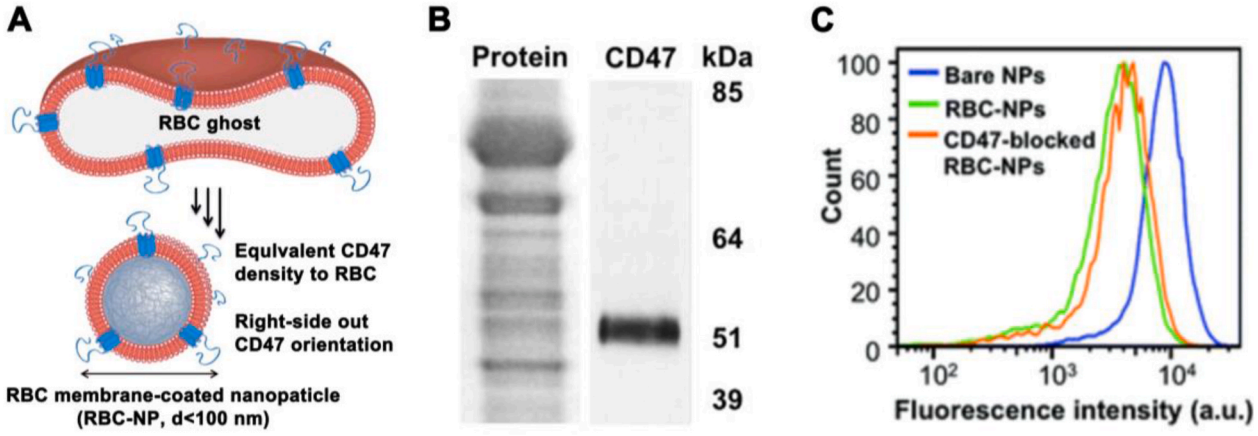

Fig. 4. Functionalization of "self-labeling" with naturally derived RBCM on nanoparticles. A) The CD47 content of RBCM-coated nanoparticles (RBC-NP) is comparable to that of natural RBCs. B. The protein content and CD47 expression on RBC-NP. C) Flow cytometry to verify the role of CD47 on RBC-NPs in evasion from phagocytosis by macrophages. Adapted with permission from Ref. [131]. Copyright © 2013 Royal Society of Chemistry.

complexes (IC) bound to CR1 on RBCs, and the transfer of fluorescently labeled IC from RBCs to human macrophages is tracked by time-lapse photography [134].

CR1 acts as a bridge for the clearance of immune complexes and an entry point for certain pathogens. Viruses and bacteria can adhere to RBCs through CR1. Therefore, RBCM can serve as an adsorbent and neutralizing agent for toxins. A biomimetic nanosponge is formed by coating RBCM onto polymer nanoparticles. The designed nanosponges decorated by RBCM (acts as a "bait") can broadly adsorb and neutralize a variety of hemolysin toxins with different structures, including melittin from bee venom, α-hemolysin from methicillin-resistant Staphylococcus aureus (MRSA), listeriolysin O from Listeria monocytogenes, and streptolysin O from Group A Streptococcus [135].

DAF, also known as CD55, is a membrane protein encoded by the CD55 gene in humans, with a molecular weight of approximately 70 kDa. DAF is a single-chain membrane glycoprotein that promotes the decay of C3 convertase. It is anchored to the cell membrane through glycosylphosphatidylinositol (GPI), a structure that may have a role in signal transduction. DAF is widely expressed on various cell surfaces, including RBCs, leukocytes, and endothelial cells [136]. The primary function of DAF is to protect host cells from complement-mediated lysis. Its mechanism includes preventing the assembly of C3 and C5 convertases of both the classical and alternative pathways, and inducing the rapid dissociation of the catalytic units C2a or Bb, thus destabilizing already formed C4 and C5 convertases, inhibiting the activation of the complement attack units. This inhibitory action of DAF is restricted to C3 and C5 convertases directly bound to cells, meaning DAF does not inhibit normal complement activators like microorganisms and immune complexes on target cells [137]. DAF can be targeted to RBCs via single-chain antibody fragments (scFv) to inhibit complement-mediated lysis of RBCs. This approach may be applicable to diseases caused by membrane protein deficiencies, such as Paroxysmal Nocturnal Hemoglobinuria (PNH) [138].

CD59, also known as protectin or membrane attack complex inhibition factor (MACIF), is encoded by the CD59 gene as a precursor protein consisting of 128 amino acids [139]. CD59 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein. The mature protein contains 77 amino acid residues and is fixed to the cell surface by a GPI anchor structure [140]. The primary function of CD59 is to inhibit the formation of the membrane attack complex (MAC) during the terminal stage of the complement cascade by interfering with the binding of C8 and C9, thereby protecting host cells from complement-mediated lysis [141]. CD59 is the only MAC-inhibitory protein on human cells and serves as the last line of defense against the terminal pathway of complement activation. PNH lacks the complement inhibitor CD59.Research has utilized a novel synthetically modified recombinant human CD59, rhCD59-P, which is a membrane-bound protein. Treating PNH RBCs in vitro with rhCD59-P brings CD59 levels to those comparable with normal RBCs and effectively protects RBCs from complement-mediated lysis [142].

One strategy for loading on the surface of RBCs involves the interaction between biotin and avidin. This application strategy is closely related to complement regulatory proteins. Highly biotinylated RBCs (b-RBCs) are prone to complement-mediated clearance, whereas moderately biotinylated RBCs are stable in serum [143]. The increased sensitivity of avidin-treated RBCs to complement lysis is primarily due to the inhibition of DAF and CD59 functions, with CD59 inhibition playing a more significant role [144].

The biotin-avidin system is prone to complement-mediated clearance. Phosphatidylethanolamine (biotin-PE) is used to link avidin to the surface of RBCs, although biotin-PE itself can enhance the classical complement pathway's lytic effect on RBCs. However, this method of attaching avidin to RBCs via biotin-PE can be used to create immunoerythrocytes. These immunoerythrocytes can specifically bind to biotinylated antibodies, effectively and specifically binding to antigen-coated surfaces. Even in the presence of soluble antigens, they remain resistant to complement lysis. This finding expands the application of immunoerythrocytes in drug targeting [145].

2.2.2.3. Glycophorin A (GPA). GPA is one of the most abundant proteins on the surface of RBCs and is one of the primary sialoglycoproteins of the RBCM. GPA features a glycosylated N-terminal segment that extends outside the membrane, containing the MN blood group antigens. GPA is a crucial component of the RBCs' cytoskeleton, playing a significant role in maintaining the stability and shape of RBCs, as well as in the expression of blood group antigens. GPA is also one of the common targets on RBCs' surface for drugs [9].

Plasminogen activators are utilized for emergency thrombolysis, limited by ineffectively targeting impermeable occlusive clots. Researches have developed a recombinant biopharmaceutical that fuses a mutated form of tissue plasminogen activator (tPA) with a single-chain variable fragment (scFv) of an antibody that specifically binds to GPA on mouse RBCs. The fused construct, referred to as anti-GPA scFv/PA, demonstrates the ability to selectively bind to mouse RBCs, thereby extending the drug's half-life and minimizing drug extravasation. Additionally, it significantly prolongs fibrinolytic activity within both arterial and venous circulation, including the delicate cerebral vascular system, thus contributing to the prevention and treatment of thrombosis [146].

2.2.2.4. Glucose Transporter 1 (GLUT1). GLUT1 on RBCs is a transmembrane protein primarily responsible for transporting glucose from the bloodstream into the interior of RBCs, which is essential for maintaining the energy metabolism of RBCs [102]. The expression of GLUT1 is regulated by the SLC2A1 gene, serving as a key functional protein in tissues and organs such as the brain, nervous system, and muscles. Structurally, GLUT1 is composed of 12 transmembrane helices that form N-terminal and C-terminal domains. The cavity between these domains orients the intracellular region, resulting in an inward-opening conformation [147].

A glucose-responsive insulin delivery system has been developed by leveraging the glucose transport mechanism of GLUT1. Insulin modified with a glucose derivative (Glc-Insulin) binds effectively to the RBCM through interactions with GLUT1, as illustrated in Fig. 5. The reversible bindings between Glc-Insulin and GLUT1 allowed for the release of insulin from the RBCs in response to hyperglycemia. This strategy emulates the function of pancreatic beta cells, maintaining glucose levels at approximately 200 mg dL^{-1} over a 24-h period. Consequently, this approach has the potential to reduce the frequency of injections and lower the risk of hypoglycemia, thereby improving the accuracy of blood sugar control for diabetic patients [148].

GLUT1 has demonstrated its potential as a site on the surface of RBCs for connecting with drugs (Glc-Insulin) in this study [148]: i) High efficiency and specificity of drug binding. GLUT1 is the predominant glucose transporter on RBCs, with approximately 500,000 to 700,000 GLUT1 molecules on the surface of each RBC (in human RBCs), providing a large number of binding sites. Modified Glc-Insulin binds to GLUT1 through glucose analogs (such as glucosamine), proving its reversible and specific binding, and can rapidly release insulin in a high-glucose environment; ii) Rapid responsiveness. GLUT1, through competitive binding with glucose, can achieve rapid release of insulin, thereby responding quickly to high blood sugar levels, mimicking the function of pancreatic β-cells. Conversely, this method also has certain limitations: i) Differences in cellular and tissue specificity. The expression of GLUT1 varies among RBCs of different species, such as in humans where GLUT1 expression is much higher than in mouse RBCs, thus results based on mouse models may not be entirely applicable to humans, especially in long-term release and biodistribution studies. Additionally, during treatment, other cells expressing GLUT1 (such as certain endothelial cells and cancer cells) may interfere with the connection between Glc-Insulin and RBCs, causing non-specific release or off-target effects; ii) Potential side effects of glucose competition. The competitive binding between glucose and modified insulin may lead to unexpected drug release in high-sugar environments. For instance, non-specific drug release issues may arise in other high-glucose tissues (such as tumors or inflamed tissues). Future research needs to further optimize drug design, reduce off-target release, and explore the actual effects in human models to achieve clinical application.

2.2.2.5. Rh17. Rh17 is a blood group antigen encoded by genes in the Rh blood group system, and it is part of the Rh blood group system [128]. It is a cell surface antigen associated with the immunogenicity of RBCs and the compatibility of the blood group system. It has significant importance in transfusion medicine because the antigens and antibodies

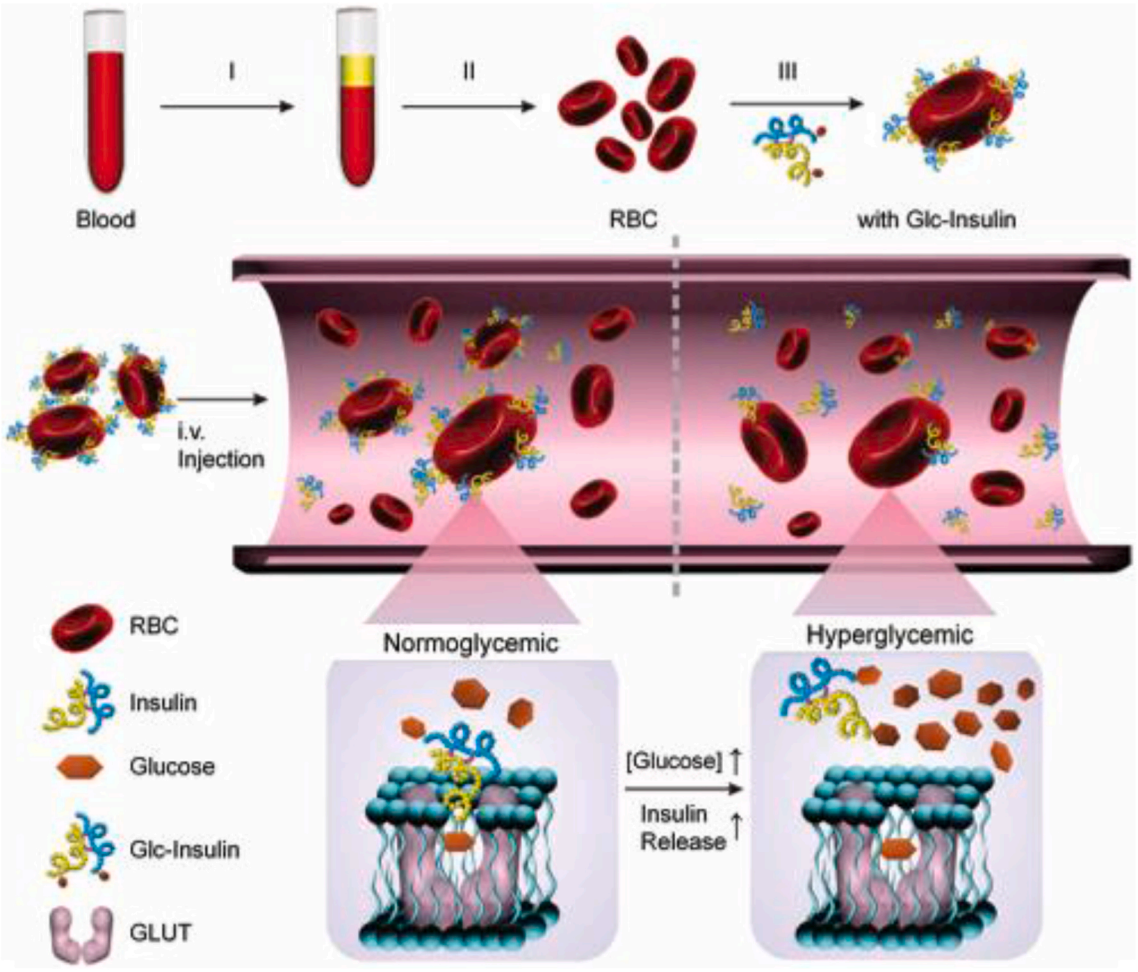

Fig. 5. Schematic diagram of an insulin delivery system based on the modification of GLUT1 on RBCM. Adapted with permission from Ref. [148]. Copyright © 2017 Wiley Online Library.

of the Rh system can cause transfusion reactions and hemolytic disease of the newborn [149].

As a potential target, Rh17 can be fused with human thrombomodulin (hTM) using scFv. Fusion proteins binding to Band 3/GPA and RhCE (Rh17/Hr0 epitopes) on RBCs can confer hTM activity to RBCs, but they have different physiological implications for RBCs. scFv and hTM-scFv targeting Band 3/GPA increase membrane rigidity and make RBCs more sensitive to hemolysis induced by mechanical stress while reducing sensitivity to hypotonic hemolysis. hTM-scFv binding to RhCE more effectively reduces platelet and leukocyte adhesion [150]. This method can enhance the circulation time and targeting of drugs in the body while reducing the immune system's recognition and attack on the delivery system, thereby improving the efficacy and safety of drugs. This approach in RBCs conjugation therapy shows target-dependent effects on RBCs physiology, providing potential for developing new therapeutic strategies [151].

2.3. RBCM-Camouflaged nanoparticles

The concept of disguising nanoparticles as RBCs or RBCM to present drug nanocarriers as "self" for prolonged circulation and reduced immune response is gaining traction. The preparation process typically involves several steps [131]: First, RBCs are lysed using hypotonic solutions to release hemoglobin, and internal components are removed through centrifugation or dialysis to obtain purified RBCM. Subsequently, these membranes are fused with nanoparticles of various purposes, such as PLGA, silica, metal oxides, or liposomal nanoparticles. Common methods include sonication, extrusion, or co-incubation techniques, ensuring the integrity of the membrane and preserving its native protein and glycosylation structures.

The advantages of RBCM-camouflaged nanoparticles (RBC-NPs) are mainly reflected in the following aspects. Firstly, their excellent biocompatibility, benefiting from the natural properties of the RBCM, can avoid strong immune responses caused by foreign bodies, thereby extending circulation time. Additionally, the native proteins (such as GLUT1) and glycosylation structures retained on the RBCM endow them with certain targeting capabilities. However, RBC-NPs also have certain limitations. Their preparation process is relatively complex, and each step from the separation of RBCM to the fusion with nanoparticles needs to be optimized to ensure stability and functional integrity. Moreover, their targeting specificity may be insufficient, mainly relying on the natural properties of membrane proteins, and the targeting capabilities for specific diseases or tissues still need further development. Individual differences are also a significant challenge, as the characteristics of RBCs may vary among different populations, which poses requirements for widespread application. Lastly, the loading capacity of the RBCM is relatively low, which may be insufficient in the context of combination therapy with multiple drugs or the delivery of large molecular drugs. Hollow RBCs' structures (Erythrocyte ghosts) that retain the morphology and some surface markers of RBCs are a type of hollow cell membrane structure with greater payload space, effectively addressing the limitations of traditional RBCM as outer coating materials with limited loading capacity, which will be discussed in detail in the following part.

3. Application of the cavitated RBCs structure in drug delivery systems

Erythrocyte ghosts (EGs), as a relatively pure cellular membrane structure, are the hollow cellular membrane structures obtained after the artificial treatment of RBCs, where the plasma membrane is ruptured and hemoglobin and other intracellular soluble proteins are removed, while preserving the morphology and some surface markers of RBCs [152]. Natural EGs are found in regions where glaucoma occurs and in aging RBCs. The permeability of EGs to small molecules (such as water and mannitol) and ions (such as chloride, sodium, and potassium) is very close to that of intact RBCs. Meanwhile, the advantages of EGs for drug loading are compatible with that of RBCs, i.e. both of which present good biocompatibility and low toxicity, strong adjustability, and reduce the risk of adverse reactions [153]. Further, the hollow structure can enhance the drug loading capacity and transport efficiency of the drug delivery platform constructed with EGs. Moreover, the high sensitivity of EGs to secretory phospholipase A2 causes the expansion of areas of the cell membrane with reduced fluidity, determining the potential of EGs in drug delivery applications [154].

The general methods for EGs-based drug loading are summarized in brief: i) The hypotonic method, which is the most commonly employed technique for drug loading, involves adding RBCs to a hypotonic solution of 0.18% NaCl at a volume ten times that of the cells. The mixture is allowed to osmose for 2 min, after which a 9% NaCl solution is added to balance the osmotic pressure. The mixture is then resealed at 37 °C for 30 min to encapsulate the drug within the ghost cells. Following encapsulation, the ghost cells are washed multiple times with a 0.9% NaCl solution to yield the drug-loaded EGs-based formulation. ii) The dielectric breakdown method: Aliquots of RBCs suspension are placed in an electrolytic discharge chamber. The dielectric breakdown experiment is conducted at 0 °C, with a peak electric field strength set at 12 kV/cm and a pulse length of 40 μs. After dielectric breakdown, an initial 10-min equilibration and resealing period is conducted at 0 °C. The ghost cells are then washed by centrifugation at 6,000×*g*, 37 °C using isotonic solution II. iii) The Triton-X-100 method: RBCs are dispersed in a hypotonic solution containing the desired enzymes and proteins, with Triton X-100 for keeping the high permeability to facilitate substrate entry into cells and Glutaraldehyde for stabilizing the cells from rupture.

The hemoglobin clearance rate for EGs can approach 100%, resulting in a final volume of 140.6 ± 15.2 fL, and facilitating an increased volume for drug payloads inside EGs [155]. EGs are emerging as promising drug carriers in therapeutic applications that necessitate a substantial quantity of drugs or the use of multiple drug combinations [156]. Throughout the transformation process, EGs undergo a significant loss of proteins, including all solutes and certain membrane proteins, which is associated with the reconstruction of the cell membrane, aimed at reducing immunogenicity and addressing other biocompatibility concerns. The elimination of solute proteins facilitates greater flexibility in drug loading and release, while alterations in membrane proteins may influence the circulation time and biodistribution of the EGs.

3.1. EGs for liver targeting

The liver serves as the largest central metabolic organ in the body, filtering blood through the hepatic sinusoidal vascular system. Liver sinusoidal endothelial cells (LSECs) and KCs, as the central to the liver's barrier function, facilitate the clearance of pathogens and dietary products from the gut, as well as various circulating degradation products, damaged cells like senescent RBCs, and toxins. KCs enhance the clearance of larger blood-borne particles through phagocytosis, while LSECs are responsible for the clearance of smaller particles (<200 μm) and macromolecules via receptor-mediated endocytosis [157]. Based on the endogenous mechanism, engineered granules acquire characteristics similar to those of senescent RBCs after modification, artificially endowed with function as a delivery system for liver targeting.

The mechanism of EG-based drug delivery system for liver targeting and retention is related to changes in membrane proteins. First, PS is primarily located on the inner side of the RBCM under normal conditions. However, during apoptosis, factors such as the decreased level of the major flipping enzyme molecule ATP11C lead to reduced flipping enzyme activity, causing PS to flip to the outer side of the cell membrane. This externalization acts as a "Phagocytic signal", which is recognized and cleared by macrophages in the liver and spleen [158]. Second, CD47 on the surface of normal RBCs serves as a "do not eat me" signal, preventing phagocytic action by macrophages. However, EGs, due to the downregulation of CD47, are cleared by phagocytosis through

KCs in the liver. Third, the Band 3 protein may undergo oxidation and cleavage as the senescence of RBCs during the EGs-processing procedure. Certain regions of the Band 3 protein, particularly amino acids 812–830, may become exposed due to conformational changes, potentially serving as a "molecular clock" for the senescence [159]. Furthermore, the oxidation and structural alterations of the Band 3 protein may influence its interactions with other proteins, such as CD47. In summary, the site-specific nature of EGs-based delivery system enables targeting to liver, thereby enhancing the enrichment of therapeutic agents in this organ and improving overall therapeutic efficacy. The macrophages in the red pulp of the spleen also play a crucial role in the physiological process of removing damaged and modified RBCs from the body. Although there are no direct data quantifying the distribution ratio of EGs in KCs in the liver and splenic macrophages, EGs have practical significance for improving the biodistribution of loaded drugs in the liver.

3.2. EGs for long-circulating

3.2.1. Combination of EGs with nanotechnology - a novel functional biomimetic delivery platform

EGs membrane-camouflaged polymer nanoparticles as a biomimetic delivery platform can not only apply for enhancing the efficacy of drug delivery but also replicate the natural behavior of RBCs. Fasudil-loaded Nano-erythrocyte ghosts (NEGs) were prepared and verified for the characteristics as inhalable carriers for drug release in the lungs, facilitating the treatment of conditions such as pulmonary arterial hypertension [160]. Another EGs-decorated nanocomposite is formed via gold nanoclusters (AuNC) and berberine (BBR) loaded by EGs, designated as AuNC@BBR@Ghost, circulating in vivo and effectively delivering the

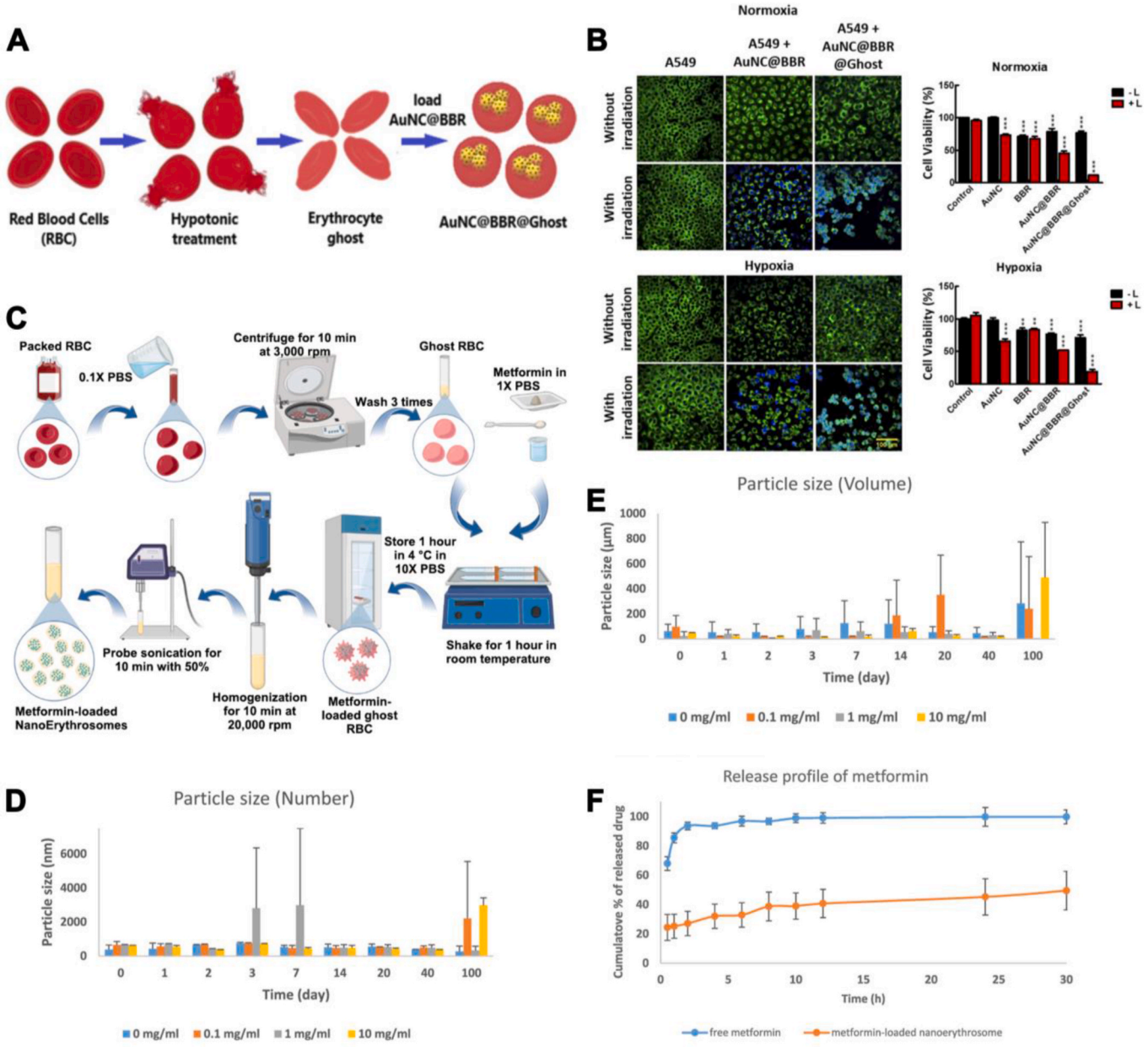

Fig. 6. Combination of Ghost with nanotechnology. A) Scheme for preparation of EGs-decorated nanocomposite, AuNC@BBR@Ghost. B) Synergistic effect of AuNC@BBR with EGs for photodynamic therapy under hypoxia on anti-tumoral treatment. A) and B) are adapted with permission from Ref. [161]. Copyright © 2024 Royal Society of Chemistry. C) Scheme for preparation of Metformin-loaded Nano-EGs via hypotonic method. D) and E) Dose- and time-dependent changes in particle size of Nano-EGs. F) Drug release profiles of metformin-loaded Nano-EGs. C-F) are reprinted and adapted from Ref. [162] with permission from Elsevier.

drug to target tissues (Fig. 6A and B). The approach of EGs-decoration improves the biocompatibility of the drug, and the residual hemoglobin in the EGs simultaneously acts as an oxygen carrier, providing the necessary oxygen for photodynamic therapy (PDT) in the hypoxic tumor microenvironment, thus synergistically enhancing the antitumor therapeutic efficiency [161].

Metformin-loaded Nano-EGs prepared by the hypotonic method can cross the blood-brain/tumor barrier (BBB/BBTB), directly delivering drugs to the brain tumor site. These Nano-EGs possess an ideal size, unique shape, ease of construction, high loading capacity, biocompatibility, and most importantly protect metformin from the influence of peripheral metabolic enzymes, prolonging its blood half-life and ensuring consistent therapeutic effects (Fig. 6C–F) [162].

3.2.2. EGs as antigen peptide delivery systems

EGs can serve as carriers for antigenic peptides in vaccine research for animals and humans. Due to their biocompatibility and tunable surface properties, EGs can effectively carry and transfer antigenic peptides to the immune system, eliciting a specific immune response without causing significant innate immune stress, thus prominently improving the safety of the vaccine [163]. Profiting from the controllable immunogenicity of EGs, plasmid DNA-loaded EGs selectively magnified the expression of the administered genes in blood. After intravenous injection of interleukin-2 (IL-2)-coded plasmid-loaded EGs in mice, the level of plasmid DNA in the blood was increased by 92,000 times compared to the injection of free plasmid within 21 min. On the third day after administration, the expression of IL-2 delivered by EGs was significantly observed in blood rather than that of other organs. Among all blood cells, a subset of granulocytes showed higher levels of IL-2 expression compared to monocytes [164]. Incorporation of coagulation factor IX into EGs can improve the stability and half-life of coagulation factor IX, and also facilitate the gradual release of coagulation factor IX in bloodstream, ultimately reducing the required dosage and frequency of treatments for patients [165]. IL-1β-loaded EGs also exhibited a half-life that is over 15 times longer than that of free IL-1β and a reduced elimination constant (CEL) by a factor of 30, allowing for sustained high levels of IL-1β activity in blood samples for up to 19 h [166]. These low immunogenic EGs with long-circulation have the potential to apply for accelerating the healing of infected wounds through localized immunotherapy, while also minimizing the risk of complications and decreasing drug toxicity.

In summary, EGs-based drug delivery systems represent a highly promising non-viral gene delivery system characterized by extended circulation time, efficient drug loading, and favorable biocompatibility. However, there are several issues that need to be addressed. The variability of EGs, as a biological carrier, poses significant differences, making it challenging to establish a universal standard. The heterogeneity of EGs might be due to the lack of a standardized preparation process and drug loading method, resulting in variations in the carrier's properties and stability, which in turn affects drug delivery and efficacy. Additionally, the challenges in storage and transportation limit the large-scale production and application of EGs. As EGs consist of aging or damaged RBCs, it not only accelerates the clearance of EGs-DDS by tissue-resident phagocytes but also faces potential attacks by components of the circulating immune system, such as the complement system. This may lead to intravascular hemolysis, resulting in pronounced nephrotoxicity or RBCs aggregation, and consequent vascular occlusion. While the loaded drug may extend the circulation time in the body and improve targeting, there is a risk of drug leakage, affecting the drug's release kinetics and efficacy.

In future research, it is necessary to design and develop new preparation techniques to reduce the variability and heterogeneity of EGs, thereby enhancing the stability and biocompatibility of the carrier. Additionally, improvements in drug loading techniques are required to minimize drug leakage and enhance the efficiency of drug release at the target site.

4. Hemoglobin as versatile approaches for drug delivery

The cytoplasmic proteins in RBCs include hemoglobin, glycolytic enzymes, glutathione, etc. Among them, hemoglobin is the most abundant protein in RBCs and is responsible for the primary physiological function of RBCs to transport oxygen and carbon dioxide in bloodstream, making it the most important cytoplasmic protein in RBCs.

4.1. The basic structure and function of hemoglobin

Hemoglobin is a tetramer consisting of two pairs of distinct polypeptide chains, typically comprising two alpha chains and two beta chains (Fig. 7). Each polypeptide chain is associated with a heme molecule that contains an iron ion, capable of reversibly binding to oxygen molecules [167]. A fully saturated hemoglobin molecule can transport up to four oxygen molecules. This structural configuration enables hemoglobin to bind oxygen in the lungs, forming oxyhemoglobin, and to release oxygen in the tissue cells, ultimately reverting to deoxyhemoglobin [168].

The primary function of hemoglobin is to transport oxygen throughout the body. The iron ion within hemoglobin, coordinated with the porphyrin structure, plays a critical role in the binding of oxygen. The binding of oxygen to iron ions forms the oxyhemoglobin (oxyHb) complex, which induces a conformational change of oxyHb from the low-affinity T-state to the high-affinity R-state and a change in electronic state leading to the contraction of iron porphyrin bonds [169]. In the alveoli, oxygen binds with hemoglobin and is transported throughout the body via blood circulation. In addition, hemoglobin is also responsible for bringing the carbon dioxide produced by tissues back to the lungs for excretion.

4.2. Hemoglobin as a carrier for drug loading and delivery

Hemoglobin leakage usually occurs in aging RBCs under physiological conditions. Hemoglobin is released from aging RBCs to extracellular microenvironment and dissociated into two 32 kDa alpha-beta dimers with potential toxicity. The endogenous defense system weakens or eliminates the toxicity of free hemoglobin by binding to haptoglobin to form the haptoglobin-hemoglobin complex [170], which can be recognized by targets or cells expressing CD163, such as macrophages,

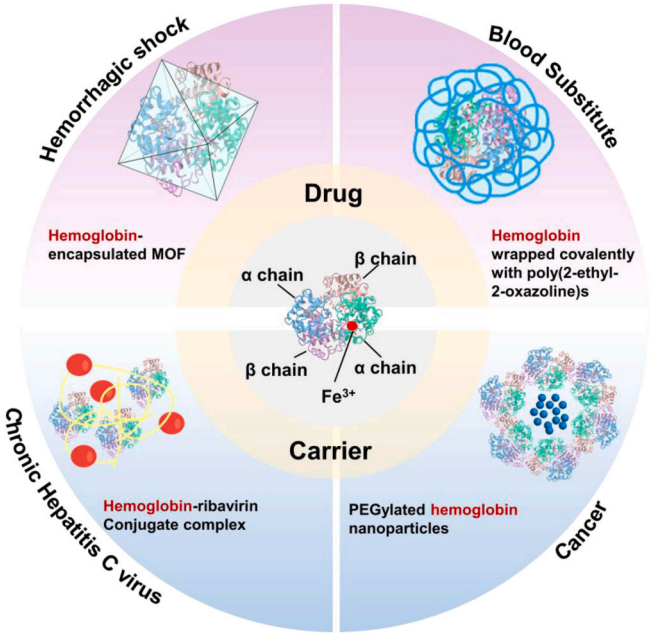

Fig. 7. Illustration of structure and novel medicinal approaches of hemoglobin.

circulating monocytes and some kinds of tumoral cells [171]. As a consequence, the physiological mechanism can be fully utilized to make hemoglobin a promising candidate as drug carrier, especially in liver diseases, macrophage-related disorders, or cancers expressing hemoglobin-related receptors, as illustrated in Fig. 7.

Drug-loaded hemoglobin nanoparticles synthesized through non-covalent interactions (mainly hydrophobic interactions) present poor stability in physiological environments (pH 7.4), especially for those administered intravenously. Surface modification by PEGylation can enhance the in vivo performance for hemoglobin nanoparticles. PEGylated hemoglobin nanoparticles loaded with paclitaxel (PTX) (PEGHb-PTX NPs) exhibit strong tumor accumulation capabilities, demonstrating enhanced cellular uptake and antitumor activity [172]. Ribavirin (RBV) and interferon-γ are commonly administered in combination for the treatment of chronic Hepatitis C virus (HCV) infection. A significant side effect associated with the treatment regimen is dose-dependent hemolytic anemia, which affects approximately 10% of eligible patients due to elevated accumulation of the drug within RBCs [173]. Hemoglobin-ribavirin (HB-RBV) conjugates sequester RBV and prevent its entry into RBCs, thereby minimizing the associated toxicity of systemic administration and augmenting the likelihood of benefit from liver-targeted therapies [174,175].

The advantages of utilizing hemoglobin as a drug carrier stem from its high biocompatibility, which minimizes the risk of immune or toxic reactions. Hemoglobin can preserve both the structure and activity of the loaded drugs, thereby enhancing the stability for both the storage and administration. The large molecular structure of hemoglobin facilitates the capacity for a broad range of multiple drug molecules and a higher drug loading efficiency. Hemoglobin-based drug carrier displayed a specifically targeting features via the binding to hemoglobin-recognized receptors on the surface of cells, thereby designing for the treatment to disease as mentioned in Fig. 7. Profiting from the intracellular degradation mechanism of hemoglobin, the release of the drug from hemoglobin-based drug carrier is triggered by endocytosis, thus presenting a controlled release characteristic and avoiding the unexpected leakage and decomposition of the loaded drugs. In conclusion, hemoglobin as a drug carrier offers significant advantages, including high biocompatibility, enhanced stability, increased drug loading capacity, targeted delivery, and controlled release, all of which contribute to improved drug efficacy, reduced side effects, and the achievement of targeted therapy.

4.3. Hemoglobin applied as blood substitutes

Blood transfusion may cause multiple risks, such as allergic reaction, HIV, hepatitis B or hepatitis C and other infectious diseases. Among them, the biggest risk may be changes in the recipient's immune function caused by blood transfusion, resulting in systemic inflammatory reactions due to cytokine release. Given the biological limitations, side effects, and storage challenges, the development of blood substitutes is imperative. Although the oxygen-carrying function of blood is primarily attributed to hemoglobin within RBCs, administration of hemoglobin i.v. directly is associated with several risks: i) Potential risk of the deposition of hemoglobin and RBCs matrix, leading to renal tubular obstruction, renal dysfunction due to heme pigment deposition, and renal toxicity stemming from reduced renal blood flow caused by hemoglobin-induced vasoconstriction; ii) Excessive oxygen transferred by hemoglobin solutions to tissues or infiltrating to the interstitial space between endothelial and smooth muscle cells, reacts with nitric oxide, resulting in the risk of vasoconstriction [176]; iii) In the absence of the allosteric effector 2,3-diphosphoglyceric acid (2,3-DPG), free hemoglobin demonstrates a high affinity to oxygen, which may result in inadequate oxygen release [177]; iv. As a natural tetramer, hemoglobin is rapidly degraded into dimers upon administration, which are then quickly filtered by the glomerulus and cleared from circulation, resulting in a shorter half-life for limiting its capacity to transport oxygen over extended periods.

The development of hemoglobin-based blood substitutes must address several challenges to ensure both the safety and effectiveness. Hemoglobin oxygen carriers (HBOCs) are a type of RBCs substitute capable of transporting oxygen, derived from hemoglobin. HBOCs can be synthesized through various chemical modifications, including cross-linking, polymerization, coupling, or encapsulation within a synthetic membrane [178,179]. These modifications aim to address the limitations associated with the direct injection of hemoglobin.

4.3.1. Conjugated hemoglobin through chemical modification

Conjugated hemoglobin, also known as surface-modified hemoglobin, refers to the conjugate product formed by the combination of hemoglobin with inert polymers. Conjugated hemoglobin possesses features of biocompatibility and prolonged retention in bloodstream. The modification of conjugation can also endow hemoglobin with enhanced molecular weight and stability of the tetramer structure, thereby alleviating allergic reactions and nephrotoxicity caused by free hemoglobin.

HES–bHb conjugate was prepared by conjugating Bovine hemoglobin (bHb, contains multiple amino groups) with oxidized hydroxyethyl starch (HES, contains multiple aldehyde groups). The aldehyde groups of a single HES molecule can react with the amino groups of hemoglobin subunits., The four subunits of hemoglobin can be cross-linked intracellularly through the conjugated HES, thereby preventing the dissociation of bHb subunits. HES–bHb conjugate was verified as an oxygen carrier with the capability for plasma volume expansion, which increases the hydrodynamic volume, colloid osmotic pressure, and viscosity of bovine hemoglobin. In terms of functionality, HES–bHb conjugate, with P_{50} value ranges from 15 to 20 mm Hg, facilitates adequate oxygen delivery to tissues and mitigates tissue hypoxia [180]. Another artificial oxygen carrier POx-Hb was designed through poly (2-ethyl-2-oxazoline) (POx) covalently encapsulating hemoglobin, and integrated physicochemical and physiological characteristics by linking the thiol terminals on the surface of pig hemoglobin with POx derivatives. The colloidal osmotic pressure and oxygen affinity of POx-Hb are slightly superior to those of unmodified hemoglobin. POx-Hb does not only exhibit comparable oxygen-binding properties to human hemoglobin, but also demonstrates favorable compatibility within the bloodstream for extending the half-life without presenting acute toxicity to associated organs [181].

4.3.2. Encapsulated hemoglobin with nanocarriers

Free hemoglobin exhibits excessive reactivity, but insufficient circulation time for aggregated hemoglobin, limiting the scope of their applications to short-term administration [182]. Magnetite nanoparticles and liposomes can serve as carriers for hemoglobin that prevent the rapid release of hemoglobin into the bloodstream while protecting the premature interactions between the internal erythroid substitutes and the external environment. Hemoglobin is concentrated on the surface of magnetite nanoparticles to form a protein crown, which effectively inhibits the dissociation of hemoglobin. Subtly, magnetite nanoparticles, which are composed of iron, are ultimately biodegraded as supplements to the body's iron pool [183]. Additionally, conjugates-encapsulated liposomes can be regard as negatively charged RBCs substitutes that mimic the behavior of natural RBCs. A phospholipid-cholesterol mixture was prepared by encapsulating a concentrated hemoglobin solution into microcapsules with approximately one micron or smaller in diameter. These microcapsules, also referred to as blood corpuscles, are sturdier than RBCs and exhibit stability even after freezing. As blood substitutes, the hemoglobin-encapsulated microcapsules possess similar oxygen and carbon dioxide loading capacities as RBCs and can be tailored to exhibit comparable electrokinetic properties, thus avoiding triggering immune responses in rats [184]. Another study demonstrated that 10 mol% polyethylene glycol of ethanolamine distearyl phosphate 5000 (PEG-PE)

as an excipient for hemoglobin-encapsulated liposomes (LEH) can significantly reduce the non-specific accumulation of LEH in liver and spleen compared to LEH without PEG, leading to the prolonged circulation time and enhanced oxygen delivery capabilities [185].

Hemoglobin holds potential as a valuable oxygen therapeutic agent in transfusion medicine, especially for the modified ones through conjugation and encapsulation. The advantages of hemoglobin as blood substitutes are summarized: i) Hemoglobin-derived blood substitutes are classified as universal blood type O negative, which avoids the risk of blood type matching and thus broadens their applicability; ii) Hemoglobin-derived artificial blood can mitigate risks associated with disease transmission and immune suppression for both doctors and patients; iii) More flexible conditions for storage unlimited by both refrigeration and the 42-day shelf life, compared to fresh blood. The improvements in functionality, safety, and convenience by hemoglobin-derived blood substitutes significantly extend the options for blood-dependent treatment for clinical use.

4.4. Other applications

In addition to serving as drug carriers or blood substitutes, hemoglobin has demonstrated various applications as promising biomaterials for cancer therapy and inflammatory diseases. For instance, the oxygen-supplying capacity of hemoglobin can alleviate hypoxia in the tumor microenvironment, enhancing the efficacy of treatments such as radiotherapy, photodynamic therapy, or sonodynamic therapy by inducing the production of reactive oxygen species [161]. Moreover, hemoglobin-related biomaterials can also be assigned for delivering various bioactive gases such as carbon monoxide and nitric oxide for the treatment of different inflammatory diseases [186].

5. Measurements of the biocompatibility, pharmacokinetics and biodistribution (PK/BD) of RBCs-derived delivery system

5.1. Preliminarily in vitro analysis of the biocompatibility

Drug delivery systems DDS that incorporate RBCs or RBCs-derived components may alter the physicochemical and biological properties of RBCs during their construction, potentially affecting the efficiency of the carrier's delivery. As reasonable initial tests of the biocompatibility of RBCs-derived DDS, preliminary in vitro analyses of characteristics such as aggregation, complement fixation, hemolysis, resistance to mechanical and other stresses, translocation of PS to the RBCs' surface, level of CD47, changes in shape, rigidity, and resistance to mechanical stress have been generally employed. For instance, comparing the physiological changes of RBCs after binding to different targets, including osmotic resistance, mechanical strength, deformability under flow, and exposure of surface PS, has been used to identify the optimal targets for therapeutic fusion proteins to bind to human RBCs [150]. In the study of delivery systems with nanoparticles attached to the surface of RBCs, the aggregation of RBCs, their sensitivity to osmotic, mechanical, and oxidative stress are appropriate indicators to assess how the properties of nanoparticles (such as material, surface charge, and loading amount) change the biocompatibility of the delivery system [187,188]. The disturbance of the complement system by RBCs-derived DDS upon entering the bloodstream is an important event concerning biocompatibility. The infusion reaction initiated upon contact of the surface of DDS with the blood's plasma proteins, particularly those proteins that activate the complement cascade. The level of complement component C3a has been detected using ELISA to assess the degree of complement activation, ensuring that DDS maintains low immunogenicity [41]. For RBCs-derived membrane components-coated DDS, determining and altering surface CD47 levels can be used to control the circulation characteristics of DDS, with high levels of CD47 for prolonged circulation and low levels for liver targeting [131,133]. These in vitro analysis methods provide researchers with fundamental parameters to efficiently design and optimize RBCs-derived DDS, allowing for qualitative and semi-quantitative comparisons.

5.2. Quantitative measurements of PK/BD in vivo

The majority of the studies on PK/BD of RBCs-derived DDS have crucial deficiencies. For example, optical methods including detection of fluorescently labeled DDS, providing important information on localization in tissues, do not provide accurate quantitative data measuring the tissue uptake in animal studies. Moreover, labeling of labile components of the DDS can lead to artifacts of their dissociation in the body. Labeling ligand moieties is prone to artifacts of tracing detached ligand leading to overestimate targeting. Noncovalent intercalation of hydrophobic labels in carriers is marred by artifacts of their redistribution in cellular membranes and other biological sinks, such as lipoproteins. Ideally, both the cargo and carrier should be stably traced by conjugated labels. The only way to do this is to trace isotope labeled RBC-drug complex in which the carrier and cargo are labeled by two distinct isotopes to detect both components of the DDS in blood and tissues for measuring PK/BD. For example, RBCs and liposomes were labeled with ^{51}Cr and ^{111}In, respectively. The detachment of liposomes from RBCs and subsequent clearance by the RES was assessed by comparing the signals of ^{111}In and ^{51}Cr [188]. In another study, ^{125}I was used for labeling membrane components or RBCs-loaded cargo, while ^{51}Cr was used for intracellular contents [189]. Dual isotope labeling provides a powerful tool for rigorous and accurate evaluation of the PK/BD of nanocarriers in vivo, especially when nanocarriers interact with cells within biological systems.

6. Biocompatibility and safety

RBCs have been recognized as excellent drug delivery carriers. Over the past few decades, several RBCs-based DDS have entered or are about to enter clinical stages. However, the therapeutic efficacy, stability, adaptability, and potential toxicity of each drug and nanoparticle are yet to be determined, which affects the biocompatibility of RBCs. To ensure the safety of RBCs-derived DDS in medical applications, relevant indicators should be closely monitored [20].

Most studies have focused on the positive aspects of drug delivery using RBCs, with few addressing potential negative impacts. Excessive modification of RBCM components in drug delivery systems may compromise membrane integrity [190]. Such modifications can accelerate RBCs clearance, diminishing their functionality as drug delivery carriers. Uncontrolled drug conjugation may direct a large number of RBCs to the RES, overloading these sentinel macrophages. This can lead to reduced immune function or hyperactivation of the immune system, potentially causing a cytokine storm [12]. Furthermore, in addition to clearance by tissue-resident phagocytes, modified RBCs are vulnerable to attacks by circulating immune system components, such as the complement system. This can result in intravascular hemolysis, causing significant endothelial and renal toxicity, or RBC aggregation, leading to vascular occlusion, ischemia, and endothelial damage [191]. Rupture of drug-loaded RBCM compromises drug delivery and may trigger a series of harmful side effects in patients, including local and systemic inflammation, vascular occlusion, endothelial cell activation, and kidney damage due to the release of free hemoglobin. In RBCs carrying nanoparticles, a high NP-to-RBCs loading ratio can affect RBCs permeability, mechanical sensitivity, oxidative hemolysis, and uncontrolled aggregation [187].

The key solutions to these issues mainly involve screening for the best indicators and avoiding RBCs damage through crosslinking of the cell membrane or other mechanisms related to the over-conjugation of therapeutic molecules to the RBCs surface. For example, when loading drugs onto the RBCs surface, appropriate targets and modification levels should be selected to balance epitope shielding and membrane deformability, thereby minimizing interference with RBCs parameters

[39,192]. Studies have shown that targeting Band 3 and GPA with single-chain antibody fragments increases membrane rigidity and makes RBCs more sensitive to hemolysis induced by mechanical stress, while reducing sensitivity to hypotonic hemolysis [150].

The properties of RBCs-associated carriers are influenced by the glycocalyx. RBCs-conjugated tPA is protected by plasma inhibitors in the RBCs glycocalyx [193]. Additionally, conjugation with RBCs carriers produces high concentrations of local tPA, allowing for localized clot dissolution near the RBCs-tPA complex. This leads to the formation of patent channels across clots in models of thrombotic vascular occlusion [20,194].

All techniques for encapsulating drugs in RBCs that have progressed to clinical stages mostly use osmotic swelling of RBCs. This involves opening pores in the RBCM to allow for the exchange of hemoglobin or drugs through a concentration gradient, then resealing the pores to return to an isotonic solution. Osmotic shock affects the integrity, plasticity, and mechanical robustness of the RBC membrane. Membrane-penetrating peptides (MPPs) cross the RBCM without creating pores or other damage, intertwining the delivered MPPs with the drug [195].

RBCs conjugated with nanoparticles should be screened through high-throughput in vitro tests to determine the optimal nanoparticle-to-RBC loading ratio [191]. For specific needs or targets, factors such as the geometric shape, plasticity, and surface characteristics of the nanocarriers, and the use of low-toxicity nanomaterials to reduce immunogenicity should be considered [196]. Coating nanocarriers with PEG or using elongated and flexible nanocarriers has been shown to slow RBCs clearance [197]. Adding albumin can reduce RBCs aggregation [191].

The complement regulatory pathways of RBCs are closely related to biocompatibility. Excessive modification of surface proteins on RBCs inhibits the activity of complement regulatory proteins, CD55, and CD59 on the RBCM [198]. For instance, when drugs are loaded onto the RBCs surface using the biotin-avidin method, excessive crosslinking of avidin leads to the formation of complexes containing anti-biotin proteins with CD55/CD59 on the RBCM, resulting in the loss of CD55/CD59 function and subsequent RBCs lysis. CD55 and CD59 are key factors in inhibiting the activation of complement component C3, which is crucial for preventing complement-mediated lysis of RBCs. In the absence of functional complement proteins, the complement pathway is activated, leading to the formation of the terminal MAC, ultimately causing RBCs lysis [199].

Biocompatibility is a crucial characteristic of RBCs delivery systems. Enhancing safety through various approaches can reduce unintended, uncontrolled adverse reactions of RBCs-DDS and lower biotoxicity.

7. Clinical applications and limitations of RBCs-derived delivery strategies

7.1. Clinical applications

The RBCs-derived DDS, as an emerging drug delivery technology, has entered clinical trials in multiple fields and demonstrated promising application prospects. A representative example is ERY-ASP (RBCs-encapsulated L-asparaginase) developed by Erytech Pharma, which encapsulates L-asparaginase within RBCs. This system prolongs the drug's half-life by shielding the enzyme's antigenic epitopes through the RBCM. Additionally, the naturally long lifespan of RBCs (approximately 120 days) effectively extends the enzyme's duration of action. Furthermore, RBCs ensure a slow and sustained drug release, thereby avoiding acute toxicity caused by high drug concentrations. ERY-ASP has been investigated for the treatment of acute lymphoblastic leukemia (ALL), demonstrating lower incidence of allergic reaction and longer time for maintaining L-asparaginase activity in a phase II/III clinical trials (NCT01518517) [200]. A phase II clinical study (NCT03267030) that employed ERY-ASP in ALL hypersensitive patients to PEG-Asparaginase was recruited for evaluating the safety and pharmacological profile. The treatment was well tolerated and most patients exhibited levels of asparaginase activity that were superior to the therapeutic target [201]. Unfortunately, Erytech Pharma has abandoned seeking approval for ERY-ASP for the treatment of hypersensitive ALL due to the changing competitive landscape [202]. In a phase II clinical trial for pancreatic cancer, ERY-ASP combined with chemotherapy significantly prolonged patients' progression-free survival (PFS). However, the phase III clinical trial of ERY-ASP for the treatment of second-line advanced pancreatic cancer patients (NCT03665441) was completed with the results of failing to meet the primary efficacy endpoint of overall survival [200,202,203]. A phase II/III clinical trial for the treatment of triple-negative breast cancer (TNBC) (NCT03674242) was also launched by Erytech Pharma and unfortunately terminated with negative results of failing to provide clinical benefit in the trial for the enrolled patients. Erytech Pharma has decided to cease further development of the same product candidate of ERY-ASP due to these unsatisfactory trials for both the pancreatic cancer and TNBC [202].

In diseases where glucocorticoids are the primary therapeutic approach, such as ataxia-telangiectasia (AT) and inflammatory bowel disease (IBD, including ulcerative colitis and Crohn's disease), RBCs-derived DDS have been explored as a potential administration method by encapsulating the dexamethasone prodrug DEX 21-P into RBCs. In a phase II clinical study, 22 AT patients received DEX 21-P-loaded RBCs treatment, which resulted in significant improvements in neurological symptoms without glucocorticoid-associated side effects [204]. The preliminary dosing data and encouraging safety and efficacy results prompted the subsequent phase III study (ATTeST) to evaluate efficacy of DEX 21-P-loaded RBCs (EryDex; EryDel, Medolla, Italy) compared with placebo, on neurological symptoms in children with AT (NCT02770807). As the largest randomized, placebo-controlled phase III trial to date, ATTeST was completed with no safety concerns including hyperglycemia, hypertension, hirsutism, Cushingoid appearance nor any treatment-related deaths reported. However, the primary efficacy endpoint was not met after the 6-month treatment in the entire study population, possibly related to delays in treatment reducing the number of participants who received treatment as outlined in the protocol, and potentially different treatment effects according to age [205]. Based on the findings from subgroup analyses from ATTeST, a randomized trial (NCT06193200) is in progress for evaluating the effect of DEX 21-P-loaded RBCs in patients younger than 10 years (aged 6–9 years). In another study, glucocorticoid-dependent IBD patients treated with DEX 21-P-loaded RBCs demonstrated that this approach is safe and feasible, effectively reducing inflammation and alleviating glucocorticoid dependence [206]. Furthermore, in a separate study, repeated infusions of DEX 21-P-loaded RBCs led to sustained symptom relief in pediatric Crohn's disease patients [207]. A phase III clinical trial (NCT01277289) was also carried out to study the potential of long-term treatment, but the trial has been terminated because of the difficulty in recruiting patients.

RTX-240, developed by Rubius Therapeutics, is genetically engineered $CD34^{+}$ hematopoietic stem cells from healthy O-type donors that co-expresses 4-1BB ligand (4-1BBL) and the interleukin-15 (IL-15)/IL-15Rα complex (IL-15TP). It is designed to stimulate the 4-1BB and IL-15 pathways, thereby activating and expanding both adaptive and innate immune responses mediated by T cells and NK cells to elicit robust anti-tumor immunity. RTX-240 has been evaluated through phase I/II clinical trials (NCT04372706) as monotherapy and combination therapy for patients with relapse-refractory acute myeloid leukemia (AML) and solid tumors [58,208,209]. However, in 2022, Rubius Therapeutics decided to cut off the development pipeline of RTX-240 due to the insufficient curacy and disappointing results [210].

7.2. Limitations for clinical translation

RBCs-derived DDS have demonstrated broad potential and advantages in clinical applications. These systems enhance drug bioavailability, reduce side effects, and enable sustained drug release. With

ongoing clinical advancements, RBCs-derived DDS are expected to become effective therapeutic strategies for various diseases. However, this field still faces significant challenges.

i) Ensuring efficient drug loading and stable release within red blood cells remains a critical technical hurdle;
ii) Further strategies are needed to enhance drug targeting and ensure long-term safety and efficacy;
iii) The large-scale manufacturing process of red blood cell-based drug delivery systems is complex, and production standardization has yet to be fully addressed;
iv) Further studies are required to investigate the metabolic pathways, toxicity, and potential adverse immune reactions of drug-loaded red blood cells in vivo;
v) As a novel biotechnological product, regulatory policies for red blood cell-based drug delivery systems require further development.

8. Conclusion and prospects

RBCs have presented a broad application prospect in drug delivery, and RBCs-based drug delivery systems have entered or are entering clinical testing. As discussed in this review, various engineering methodologies have broadened the utility and multifunctionality of RBCs-derived components as drug delivery systems, providing several advantages over conventional nano- and micro-drug platforms, including enhanced circulation longevity, targeted delivery to disease sites, improved immune activity, and reduced toxicity. This review highlights the unique flexibility of RBCs derivatives in the design of novel nanotherapies and nano-vaccines, particularly in the formulation of ghost-based preparations. With the advancement of biotechnology and nanotechnology, RBCs-derived delivery systems have gradually evolved into various forms, including intact RBCs, RBCM, EGs, and hemoglobin, among other different derived components. These delivery systems have demonstrated a concept of gradual evolution in terms of structural optimization, functional diversity, and application scope, while also facing challenges in the development of new technologies and practical applications. From intact RBCs to RBCM, EGs, and hemoglobin, the evolutionary process of RBCs-derived delivery systems reflects a transition from complexity to simplification, and from single-function to multifunction integration. The core concept of this process is to separate and optimize different functional modules of RBCs to construct more efficient, precise, and diverse delivery systems. Firstly, by extracting specific functional modules of RBCs (such as the immuno-stealth properties of the membrane and the oxygen-carrying capacity of hemoglobin), functional specialization is achieved. Secondly, by combining the advantages of different components and using assembly or composite technologies, multifunctional synergistic delivery can be realized, such as the simultaneous delivery of drugs and genes. Lastly, through engineered modifications, the delivery system is endowed with higher targeting and adaptability. Although RBCs-derived component-based delivery systems show great potential, their future development still needs to overcome many challenges.

i) Large-scale production and quality control: How to achieve the scaled preparation of RBCs-derived components and ensure consistency and functional stability is a key issue for industrialization;
ii) In vivo safety and long-term stability: Ensuring the stability and safety of the delivery system in the complex in vivo environment, avoiding adverse immune reactions or other side effects;
iii) Optimization design of multifunction integration: How to balance the complexity in multifunctional design with delivery efficiency will be a focus of research in the next phase;
iv) Clinical translation and regulatory adaptability: From laboratory research to clinical application, further exploration of its effectiveness and safety in humans is needed, and it must meet relevant regulatory requirements.

With the further development of nanotechnology, biomaterials science, and molecular engineering technology, delivery systems based on RBCs-derived components will provide more efficient solutions for precision medicine, personalized therapy, and comprehensive treatment. Strategies for reasonable utilization of RBCs-derived components as biomimetic functional materials present a potential solution for clinical drug delivery systems, providing an attractive option for addressing some of the most significant challenges faced in the field of biomedicine.

CRediT authorship contribution statement

Hangbing Liu: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Yi Li:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Yuli Wang:** Writing – review & editing, Visualization. **Liying Zhang:** Visualization. **Xiaoqing Liang:** Visualization. **Chunsheng Gao:** Supervision. **Yang Yang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Ethics approval and consent to participate

The current work is review-type article that do not involve any human participants or animal subjects. Ethics approval and consent to participate are not applicable.

Declaration of Competing Interests

There are no conflicts of interest to declare.

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