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Chapter

Application of Red Cell Membrane in Nanobiotechnology

Insu Kim, Gyudo Lee and Dae Sung Yoon

Abstract

Red cells are full of unique biological properties such as immune evasion and molecular-specific permeability. These properties originate from various membrane proteins on the surface of the cell membrane. For this reason, red cell membrane is coated on nanomaterials or sensors to bestow the functionalities of the membrane proteins. In this chapter, various types of membrane proteins of red cell and its functions are described. Also, the following two experimental procedures are summarized: (I) the extraction of red cell membrane containing membrane proteins and (II) coating of the extracted cell membrane onto the nanoparticles and solid surface of sensors. Finally, the applications of red cell membrane in drug delivery system and biosensor are discussed.

Keywords: red cell membrane, membrane proteins, cell membrane coating, biosensors

1. Introduction

Red cells (RCs), also called as erythrocyte, are the most abundant cells in the body and highly specialized in gas transportation [1]. RCs deliver oxygen from the lungs to all body tissues and carry carbon dioxide to the lungs. For the delivery of the gases, RCs circulate through blood vessels to the whole body without being trapped inside narrow capillary vessels (5–10 μm in diameter) which are smaller than the size of RCs [2]. Also, RCs do not get attacked by immune system such as mononuclear phagocytic system (MPS) and complement system (CS) [3, 4]. The unique characteristics of the RCs come from specialized microstructure of RC. The microstructure of RC has a biconcave disc-like shape and is fully packed with hemoglobin instead of nucleus and intracellular organelles. Also, various membrane proteins and carbohydrates are embedded in the cell membrane which characterizes intrinsic functionalities of RCs [5]. The biconcave disc-like shape maximizes the surface area of the cell, which increases the gas exchanges between internal and external gases. Hemoglobins are oxygen-transport metalloproteins that bind gases such as oxygen and carbon dioxide. Especially, membrane proteins of RC are responsible for numerous characteristics such as permeability to specific molecules (e.g., glucose, urea, and gases), immune evasive properties, unique biconcave disc-like structure, flexibility, and deformability [4, 6]. Recently, it is reported that membrane proteins of RC can be utilized by manipulating the cell membrane [7]. The RC membrane (RCM) inside the whole blood of mice or human contains membrane proteins, which were extracted and purified. Accordingly, intriguing

researches were conducted together. For example, just like RC does, RCM-coated nanoparticles showed long circulation in the blood by evading immune responses such as MPS and CS [7, 8]. Also, RCM-coated glucose sensors showed high perm-selectivity to glucose [9]. As a result, the sensor was barely affected by interfering molecules such as saccharides and antioxidants. Likewise, the utilization of the functionalities of membrane protein of RC advances nanobiotechnology in the field of drug delivery system and biosensor. In this chapter, we investigate the various membrane proteins expressed on RC and its functionalities. The techniques for extraction and functionalization of cell membrane have been researched. Also, we discuss about the application of RCM functionalization for the last decade.

2. Membrane proteins of red cell membrane and its functionalities

Membrane proteins are essential components allowing specific functionalities for cells. There are three categories classified by its function. Membrane proteins perform as receptors, transporters, and cell adhesion molecules. **Table 1** represents major membrane proteins on RCM classified by their function [5].

2.1 Membrane receptors

Membrane receptors are one of integral membrane proteins. They mediate cell signaling via binding extracellular molecules. Specifically, membrane receptors allow communication between the cell and external environment. Hormones, cytokines, cell adhesion molecules, and immunoproteins are examples of the extracellular molecules. The ligand bound of the membrane receptor may induce changes in the metabolism or activity of the cell. In RC, CD55 (decay-accelerating factor) and

Protein	Gene	Function
Membrane receptor		
CD55 ([a]DAF)	CD55	Decay-accelerating factor that prevents the activation of complement system
CD59	CD59	[b]MAC-inhibitory protein that prevents complement membrane attack complex
Transporter		
AE1 (band 3)	SLC4A1	Anion transporter
RhAG	RhAG	Ammonia transporter
Nucleoside transporter	SLC29A1	Nucleoside transporter
Urea transporter	SLC14A1	Urea transporter
Glucose transporter	SLC2A	Glucose transporter
Cell adhesion molecule		
CD47	CD47	"do not eat me" signal protein that interacts with [c]SIRPα to inhibit phagocytosis

Membrane receptors consist of CD55 and CD59; transporters consist of AE1, RhAG, nucleoside transporter, urea transporter, and glucose transporter; cell adhesion molecule consists of CD47.
[a]DAF, decay-accelerating factor.
[b]MAC, membrane attack complex.
[c]SIRPα, signal regulatory protein alpha.

Table 1.
Various membrane proteins in RCs [5].

CD59 are well-known membrane receptors which inhibit CS preventing hemolysis (**Figure 1**) [10]. In detail, CS is composed of proximal and terminal complement (**Figure 2**) [4]. The proximal CS has three pathways converged at the step of complement component 3 (C3) activation. The terminal complement is initiated with complement component 5 (C5) and ended with formation of membrane attack complex (MAC). In this cascade, CD55 inhibits C3 activation by deactivating C3 convertase [11]. CD59 inhibits terminal complement activation by preventing the formation of MAC that starts with the activation of C5 [12]. The absence of CD55 and CD59 may lead to hemolysis of RC via complement activation [4].

2.2 Transporters

Transporters are involved in the movement of specific molecules or ions across cell membrane. The proteins are involved in the movement of molecules by active transport or facilitated diffusion. It is revealed that anion, gas, nucleoside, urea, and glucose transporters are expressed on the RC. In detail, (AE1, also called band 3) is responsible for mediating the exchange of chloride ion with bicarbonate (HCO_3^-) across RCM [13]. Rh-associated glycoprotein (RhAG) is a gas transporter which permeates carbon dioxide [1]. Nucleoside transporter mediates the transport of nucleoside substrates like adenosine [14]. Urea transporter is specialized in urea transportation, which is activated by antidiuretic hormone (vasopressin) [15]. Glucose transporter (GLUT) is a uniporter that transports glucose toward intracellular orientation (**Figure 3**) [16]. GLUT is an essential protein for glucose uptake of the cell by catalyzing facilitative diffusion. Especially, RC expresses a large number of GLUT compared to other cells because the cell lacks mitochondria and the energy is produced by glycolysis of glucose [17].

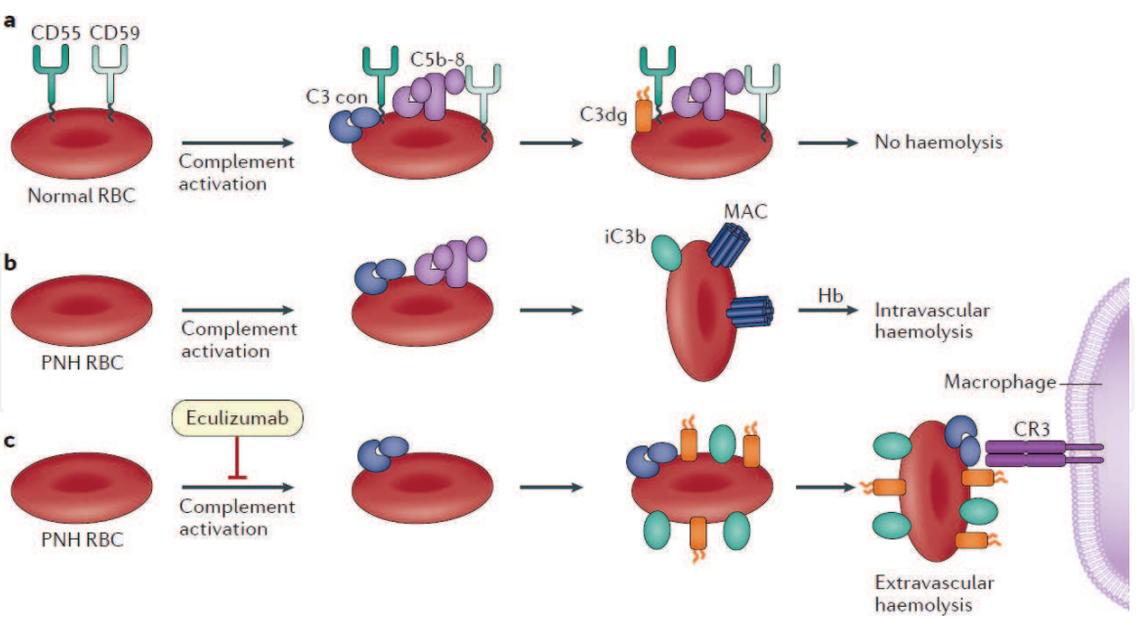

Figure 1.
*Hemolysis mechanism of paroxysmal nocturnal hemoglobinuria (PNH) via the complement system [4]. (a) Normal RBC possesses CD55 and CD59 which are glycosylphosphatidylinositol (GPI)-anchored self-protective complement regulatory factors. CD55 is a widely expressed membrane protein that accelerates the decay of C3 convertases. CD59 is the major inhibitor of terminal complement, which blocks the generation of the membrane attack complex (MAC). (b) Intravascular hemolysis of PNH RBC through C3 convertase and MAC. (c) Extravascular hemolysis of PNH RBC via macrophage. Eculizumab inhibits the complement activation by compensating CD59. *PNH, a life-threatening disease characterized by destruction of RBC by complement system; eculizumab, a monoclonal antibody complement inhibitor which is highly effective for PNH; C3 con, C3 convertase; C5b-8, complex of C5b, C6, C7, and C8 proteins; C3dg, a fragment of C3 protein, which is ligand of integrin (CR3) on macrophage; iC3b, inactivated C3b; Hb, hemoglobin; CR3, complementary 3.*

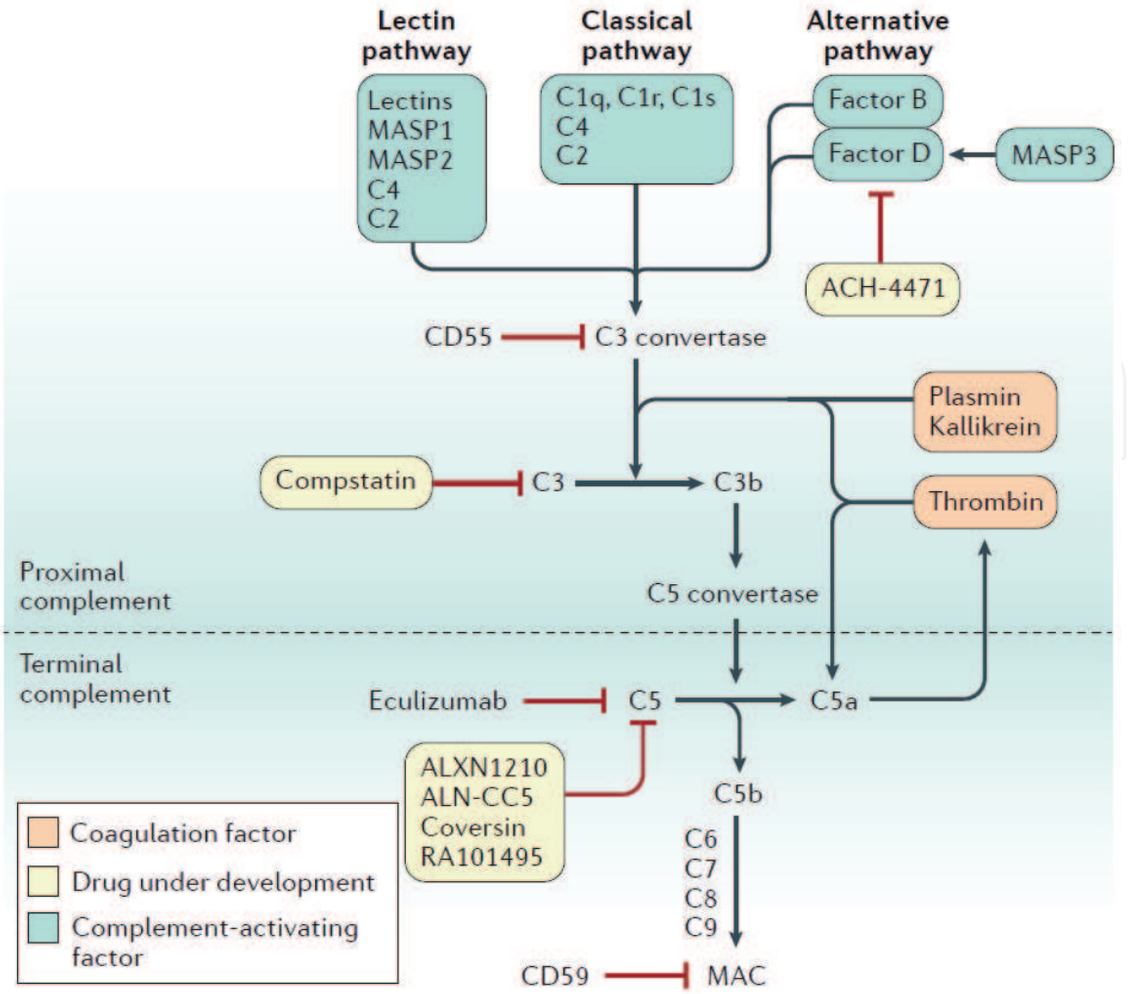

Figure 2.
Complement system signal cascade [4]. Proximal complement consists of three pathways. The lectin, classical, and alternative pathways initiate and converge at the step of complement component 3 (C3) activation. Terminal complement is initiated by C5 convertases, leading to cleavage of C5 to C5a and C5b. C5b oligomerizes with C6, C7, C8, and multiple C9 molecules to form the membrane attack complex (MAC). The CD55 inhibits proximal complement activation by accelerating the decay of C3 convertases preventing the incorporation of C9 into the MAC.

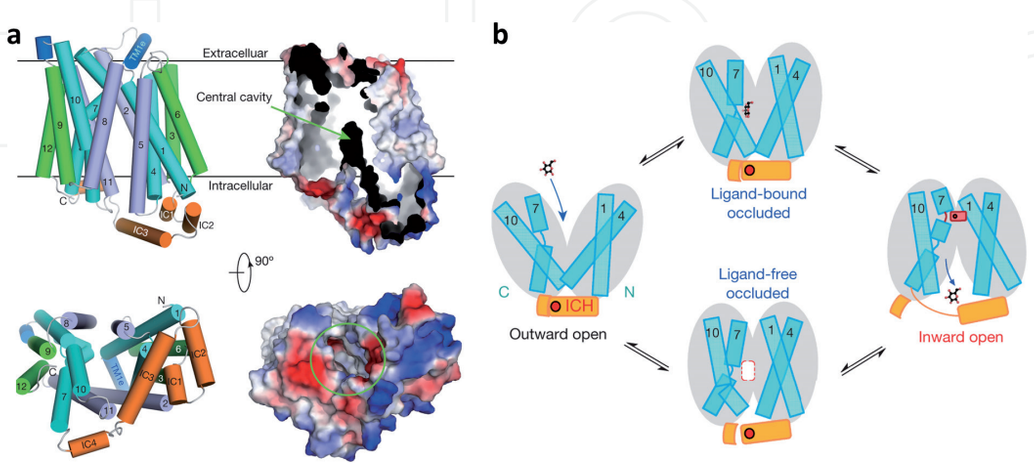

Figure 3.
(a) Overall structure of human glucose transporter-1 (GLUT1) and (b) working model for GLUT1 [16]. The working model is predicted to have four conformations (outward open, ligand-bound and occluded, inward-open, and ligand-free and occluded) required for a complete glucose transport cycle. Intracellular helix (ICH) domain of GLUT1 has two critical roles. First, the ICH domain appears to maintain a defined conformation with respect to the N domain. Second, the ICH domain is pulled toward the C domain during the inter-domain rotation from occluded to inward-open conformation.

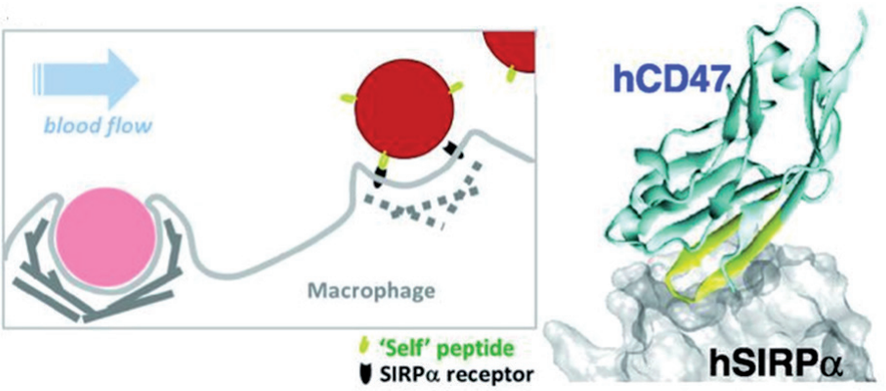

Figure 4.
The cocrystal structure shows human CD47 (hCD47) with human signal regulatory protein α (hSIRPα). The left panel depicts that the occurrence of phagocytosis depends on the reaction between hCD47 and hSIRPα [6].

2.3 Cell adhesion molecules

Cell adhesion molecules interact with membrane receptors of various cells. RC has CD47 as a cell adhesion molecule [18]. CD47 belongs to the immunoglobulin superfamily and sends a "*don't eat me*" signal to MPS such as monocyte and macrophage (**Figure 4**) [6]. This intriguing signal is derived from the interaction between CD47 and signal regulatory protein alpha (SIRPα) which is expressed on monocytes and most of subpopulations such as macrophages. Indeed, CD47-eliminated RCs were easily phagocytosed by macrophages unlike RC with CD47 [3, 19].

3. Red cell membrane extraction and functionalization techniques

3.1 Red cell membrane extraction procedure

RC can easily be extracted from whole blood of mice or human. Normally, the whole blood is extracted at vacutainer tube (evacuated tube) containing anticoagulant such as heparin, citrate, or ethylenediaminetetraacetic acid (EDTA) according to the purpose (**Table 2**). Heparin collection tubes are preferred for peripheral blood in cytogenic studies. Heparin activates antithrombin III which deactivates thrombin and serine endopeptidase, which are essential enzymes for coagulation [20]. Citrate collection tubes are employed for blood transfusion and coagulation assays because citrate reversibly binds to calcium which is an essential molecule in many steps of coagulation cascade [21]. EDTA collection tubes are usually used for complete blood count (CBC) test because EDTA is a strong anticoagulant that irreversibly binds to calcium [22]. Since coagulation requires RC to form blood clot, EDTA collection tubes are mostly used for RC extraction [7, 8, 23].

The procedure for extracting RC from whole blood is as follows [23]. Whole blood withdrawn from mice or human is centrifuged at 800–1000 g for 5 min at 4°C in order to remove the plasma and the buffy coat. The resulting sediment is washed three times with ice-cold 1× PBS to remove blood proteins adsorbed on RCs. To extract cell membrane from RCs, hypotonic treatment, homogenize, or sonication is conducted for hemolysis. Hypotonic treatment is the most convenient hemolysis procedure without disruption of membrane and membrane proteins. Washed RCs are suspended in 0.25× PBS for 20 min at 4°C. As hemolysis progresses, hemoglobin is released from RCs, and RC ghosts (empty cell membrane without cytoplasmic contents) are formed [24]. As a result, RC ghost can be verified with phase contrast

Anticoagulant	Usage	Mechanism
Heparin	Cytogenetic studies	Activate antithrombin III which deactivates serum clotting factors (factors [a]IIa and [b]Xa)
Citrate	Coagulation assays, blood transfusion	Bind to calcium reversibly (not as strong as EDTA)
Ethylenediaminetetraacetic acid (EDTA)	molecular genetic studies, complete blood counts	Strongly bind to calcium irreversibly. The absence of calcium

[a]*Factor IIa, thrombin.*
[b]*factor Xa, serine endopeptidase.*

Table 2.
Vacutainers with various anticoagulants.

microscope. To remove the hemoglobin, hemolyzed solution is centrifuged and washed. Then the RCM is collected with light pink pellet. The resulting RCM concentrate is stored in −70°C before use.

3.2 Red cell membrane coating techniques on nanoparticle and solid surface

Cell membrane coating technology comes from phospholipid manipulation technique which forms liposome and solid-supported lipid bilayer because the phospholipids are the main component of cell membrane. Although the phospholipid manipulation technique was developed quite a while ago, cell membrane coating techniques on nanoparticles were first reported in 2011 (**Figure 5**) [7]. Researchers prepared RCM-coated nanoparticles by RCM vesicle-nanoparticle fusion [25, 26]. Specifically, the extracted cell membrane was adequately diluted (membrane extraction was described in Section 3.1.). For the membrane vesicle derivation, outer forces are applied to the diluted membrane. Sonicating the membrane is the easiest way to make membrane vesicle, but it is hard to regulate the size of the vesicle. Alternatively, extrusion methods can control the size of the membrane vesicle by porous polymer membrane with various pore sizes. Next, membrane coating onto nanoparticles is conducted with the same procedure to

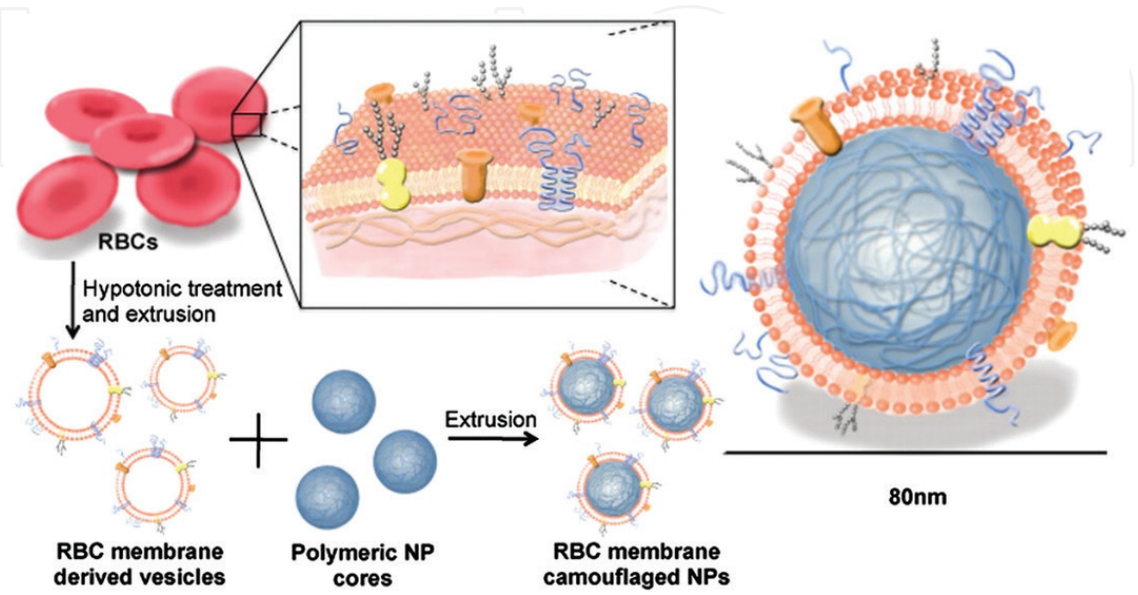

Figure 5.
The schematic illustration of the preparation of RBC membrane-coated polymeric nanoparticles [7]. RBCs, red blood cell; polymeric NP, polymeric nanoparticle such as poly(lactic-co-glycolic acid) (PLGA) nanoparticle.

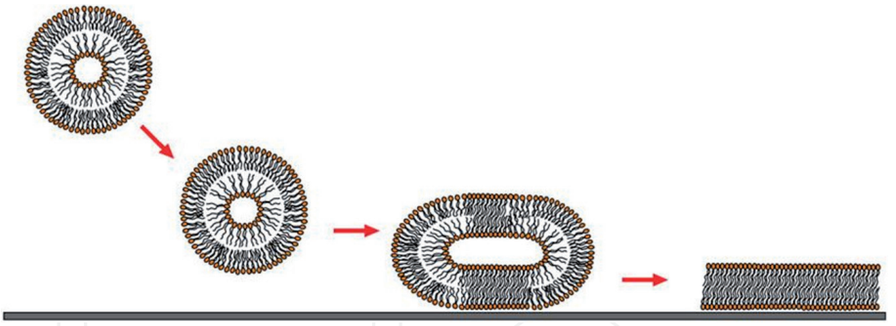

Figure 6.
Fusion of a lipid vesicle on solid surface [27].

vesicle formation (i.e., sonication or extrusion). In detail, the prepared membrane vesicles are mixed with nanoparticles. Then the sonication or extrusion of the mixture can lead to the coating of membrane onto the nanoparticles by the principle of vesicle fusion.

The cell membrane coating on solid surface is similar to that on nanoparticles described above. The coating procedure is based on vesicle fusion method (**Figure 6**) [27]. The only different procedure for solid surface coating is that thermal energy is employed instead of mechanical energy (i.e., sonication or extrusion) [9, 28]. Specifically, the cell membrane vesicles with adequate concentration were placed on solid surface and incubated for 45 min at 50°C. The thermal energy induces the membrane vesicle to collide and fuse onto the solid surface. It is noted that the temperature for vesicle fusion should be lower than the denaturation temperature of proteins to avoid the deactivation or misfolding by high thermal energy [29].

4. The applications of red cell membrane in drug delivery systems and biosensors

RCM-coated nanoparticles, also called RC-camouflaged nanoparticles, have been developed for drug delivery system since they were devised by Zhang and his group in 2011. It was found that immune evasive properties of RCM-coated nanoparticles are superior to conventional nanoparticles. The membrane proteins confer the advantages of the immune avoidance properties described in Section 2. RCM coating has been applied to various core nanoparticles such as gold, poly(lactic-co-glycolic acid) (PLGA), silica, and iron oxide nanoparticles [30]. Also, RCM can be utilized as permselective filter for glucose biosensor taking advantage of GLUT on RCM [9].

4.1 Drug delivery with red cell membrane-coated nanoparticles

In drug delivery system related with nanomaterial, long-term circulation of nanoparticles in vivo is one of the most important characteristics because various immune responses clear the foreign molecules in the body and blood [31]. Especially, MPS and CS are major immune systems eliminating drug delivery carriers. Conventionally, to evade the immune systems, the drug carriers are functionalized with polyethylene glycol (PEG) which slows clearance in blood and avoids non-specific binding of blood proteins [32, 33]. In our body, however, there is an anti-PEG immunological response which removes PEGylated nanoparticles [34]. By contrast, the RCM-coated nanoparticles showed prolonged circulation in blood. The result is exactly attributed to membrane receptors and cell adhesion molecules,

which were abundant and diverse on RCM. In this regard, the immune evasive properties of RCM-functionalized nanomaterials have great potential as clinical drug delivery carriers. In particular, it is researched that the RCM-functionalized nanoparticles showed good dispersion stability in serum and great biodistribution in mice model up to 72 h (**Figure 7**) [7]. Indeed, it is demonstrated that the RCM inhibits macrophage uptake. RCM-coated gold nanoparticles showed ~4 times higher immune evasive properties than bare gold nanoparticles [8].

4.2 Permselective glucose sensing with red cell membrane-coated biosensors

In biosensors, not only sensitivity but also specificity (selectivity) is important because numerous molecules coexist in the biological samples that may interfere the detection [35]. For example, blood contains ions, saccharides, proteins, and blood cells which hinder accurate glucose detection. Enzymatic glucose biosensors, most widely used, employ glucose oxidase or glucose dehydrogenase

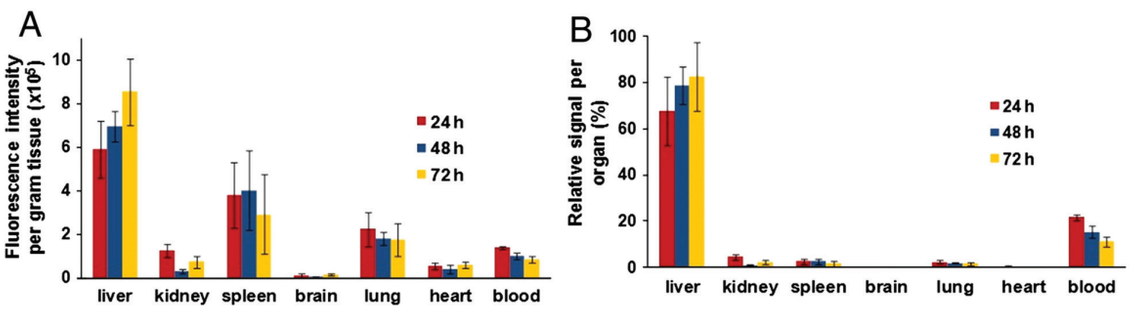

Figure 7.
Biodistribution of RCM-coated gold nanoparticles in mice [7]. The fluorescently labeled nanoparticles were injected intravenously into the mice. The fluorescent intensity at the liver, kidney, spleen, brain, lung, heart, and blood was measured at 24, 48, and 72 h. (A) fluorescent intensity per gram of tissue (n = 6). (B) Relative signal per organ.

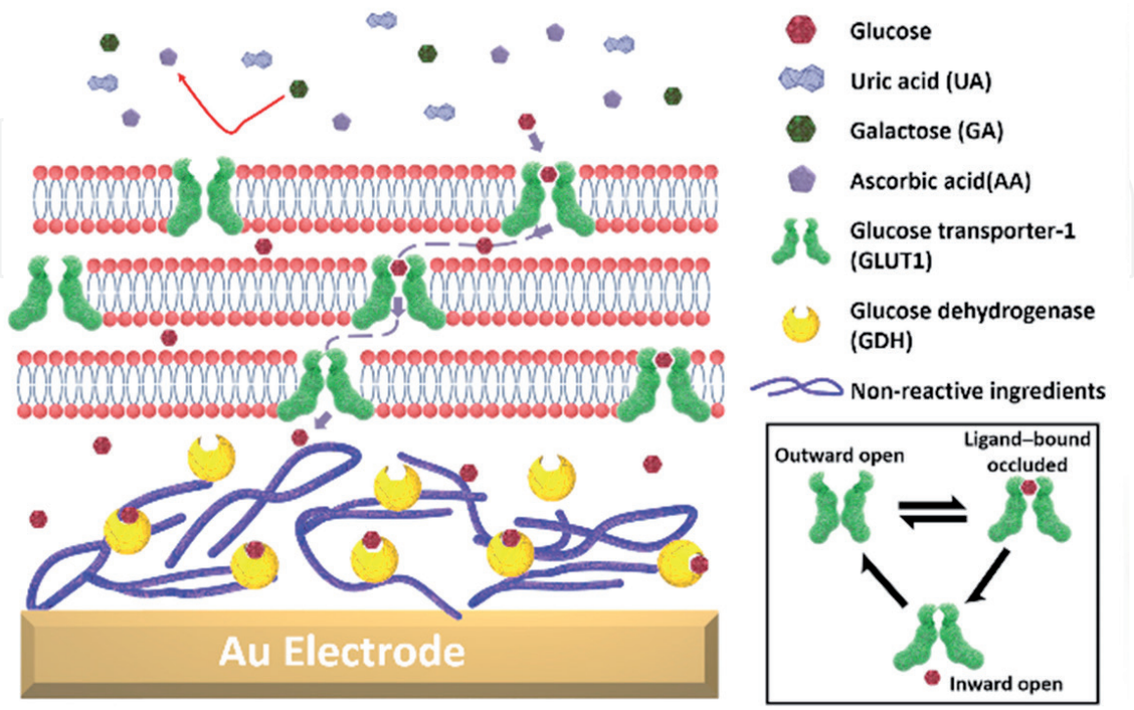

Figure 8.
Schematic illustration of RCM-coated glucose sensor [9]. The RCM-coated enzymatic glucose sensor specifically reacts with glucose via taking advantage of glucose transporter-1 (GLUT1).

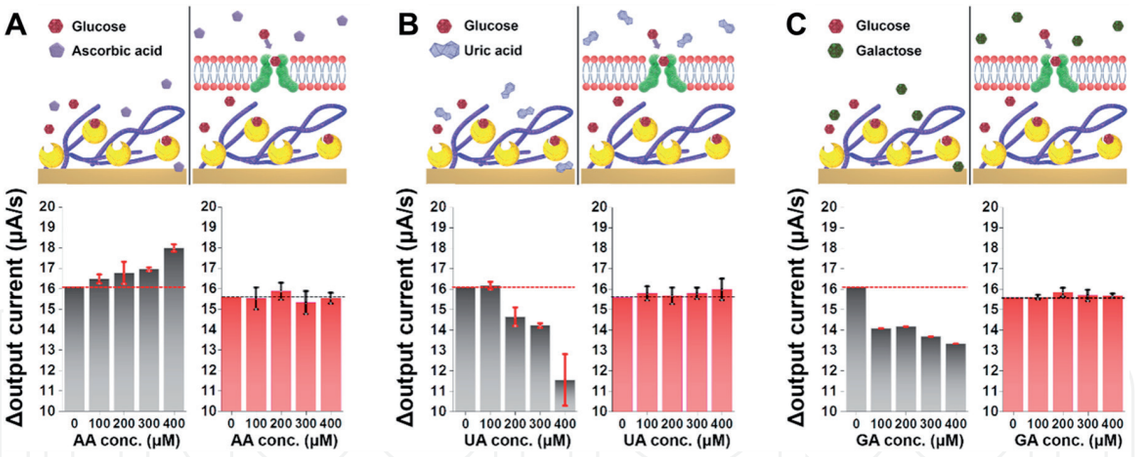

Figure 9.
Selectivity test of RCM-coated glucose sensors under competitive interactions between glucose and each interfering molecule [9]. The selectivity test was conducted with 5 mM of glucose blended with each interfering molecule, e.g. (A) ascorbic acid (AA), (B) uric acid (UA), or (C) galactose (GA). The black and red bars represent the output signal of uncoated sensor and RCM-coated sensor, respectively.

for selective detection of glucose. However, the enzymes react with glucose and other similar structured molecules (mono- and disaccharides) such as fructose, galactose, and maltose in blood. For this reason, glucose sensors are interfered by the molecules. It is reported that RCM which has glucose transporter was employed as glucose-selective permeable membrane by taking advantage of GLUT (**Figure 8**) [9]. The RCM-coated sensor showed high selectivity to glucose compared to uncoated sensor. In detail, the uncoated sensors are highly affected by the increment of interfering molecules (e.g., ascorbic acid, uric acid, and galactose), whereas the RCM-coated sensors exhibit consistency in glucose detection. In particular, RCM-coated sensor showed that the signals of glucose with interfering molecules barely change from that of glucose without interfering molecules (**Figure 9**) [9].

5. Conclusions

The RCM has various types of membrane proteins such as membrane receptor, transporter, and cell adhesion molecules. Each type of membrane proteins is full in potentials to be applied in various fields such as drug delivery system and biosensor. The well-evolved functionality of membrane proteins can be easily utilized by coating the RCM on nanomaterial and solid surface of sensors. Currently, drug delivery system is the major field of RCM application because the membrane can confer the immune evasive properties of RCM to the nanomaterials. In the future, it is expected that the RCM will be increasingly applied in development of highly selective biosensors utilizing various transporters on RCM.

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