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 REVIEW

Unveiling the Complexity of Red Blood Cells: Insights into Structure, Properties and Functions

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Abstract. *Considering the basic function of red blood cells (RBC, erythrocytes) as carriers of oxygen and carbon dioxide throughout the bloodstream, as well as their possible secondary activities, RBCs deserve more consideration. The current work attempts to serve as a summary of RBC properties, both well-established and less well-established, with a focus on pathologies and drug interactions. This review is especially important given the recent trend of employing erythrocytes as vehicles for targeted medication delivery.*

Keywords: red blood cells, functions, pathology, interactions with drugs

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1. Introduction

Human blood is formed by plasma and a variety of cells, namely white (*leukocytes:* neutrophils, lymphocytes – B and T cells, monocytes, basophils, eosinophils and macrophages), red (*erythrocytes*) and platelets (*thrombocytes*) [1]. Although all blood components are quite complex, with new information being continuously discovered, they all have their basic functions. While less than 1% of the entire blood content, leukocytes aid in fighting against infections and disease [2]. Platelets, on the other hand, help stop bleeding by interacting with blood

clotting proteins [3]. Last, but not least, red blood cells (RBCs) are the predominant cells within blood, making up to 40-45% of its volume, being quite simple in structure and possessing no internal organelles [4]. For a better understanding, Figure 1 presents a graphic representation of blood composition.

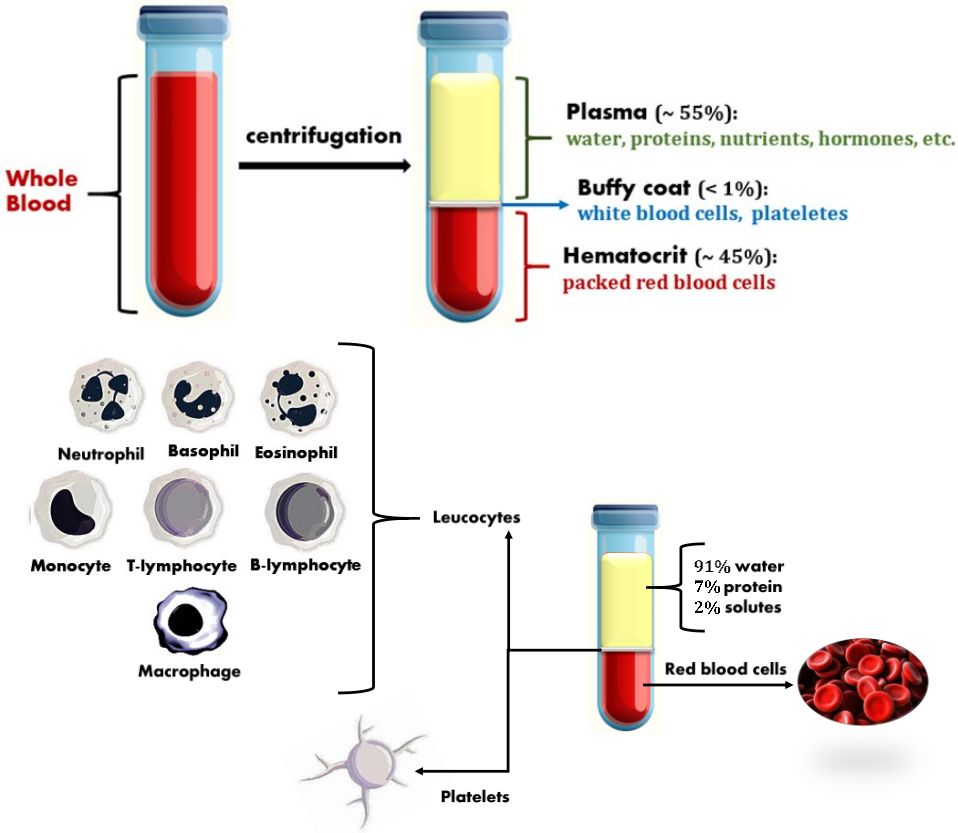

Figure 1. Blood composition

Blood components can interact with one another and are extremely dynamic. Erythrocytes, for example, can influence the movement of white blood cells by excluding them from the bulk solution [5]. It was demonstrated that the shape of erythrocytes affects their ability (through organized aggregation) to induce the radial migration of white blood cells. RBCs also interact with platelets, leading to the latter's concentration being higher towards the wall of the blood vessel. This type of interaction can be either of a turning (platelets do not make direct RBC contact and turn around in the direction they came from) or a crossing type (platelets roll over erythrocytes and proceed with their way, leading to a mild RBC deformation) [6]. Macrophages are deeply connected with red blood cells, being involved in both erythropoiesis (development and differentiation of RBCs

in the bone marrow) and erythrophagocytosis (removal of defective RBCs) [7]. As it would be impossible to convey, in a concise manner, all in depth aspects regarding every single blood component, specific functions and interactions, this review will focus on conveying some aspects regarding red blood cells.

2. The basics on red blood cells

The basic function of RBC is the transportation of oxygen from the lungs towards tissues as well as carbon dioxide back to the lungs while travelling around 300 km within the bloodstream [8]. This is achieved through the hemoglobin (Hb) which is a tetramer comprised of four polypeptide globin chains. The three types of hemoglobin (HbA, HbA2, HbF) are derived from the four types of globin chains, namely α, β, γ, δ, with each subunit containing an iron atom bound to a heme molecule [9].

The transportation of gases is due to the iron, with each hemoglobin having the ability to transport about four molecules of either carbon dioxide or oxygen. Most carbon dioxide, however, is transported as bicarbonate ions (HCO_3^-), where the H_2CO_3 deprotonation in water is a result of the CO_2 and H_2O reaction, which is mediated by carbonic anhydrase [10]. Nonetheless, RBCs confront some obstacles even though their role is quite straightforward. These biconcave disks with a thickness and diameter of around 2-2.5 μm and 7-8 μm, respectively, must be able to squeeze through capillaries as small as 3 μm in diameter, as well as through slits of less than 1 μm in width in the reticuloendothelial system [11]. Their high plasticity is a crucial parameter to ensure that oxygen is properly delivered. Any disturbance in the shape-maintaining ability of RBCs would lead to hemolytic anemia. The deformation property of red blood cells is of course in part due to their unique biconcave shape, but also the composition and organization of the cell membrane (52% protein, 40% lipids, 8% carbohydrates), namely cytoskeleton and lipid bilayer [12, 13]. There are many intricacies concerning the structure of the erythrocyte membrane, but briefly, the lipid bilayer is composed of phospholipids, namely phosphatidylethanolamine and phosphatidylserine (inner layer) and phosphatidylcholine and sphingomyelin (outer layer) with the intervening space being occupied by cholesterol [14]. On the other hand, spectrin, ankyrin, protein 4.1R, actin, as well as actin associated protein form the RBC cytoskeleton which binds to the bilayer through protein complexes [15]. Given that the precise position of several components is still unknown [15], Figure 2 provides a simplified schematic representation of these aspects.

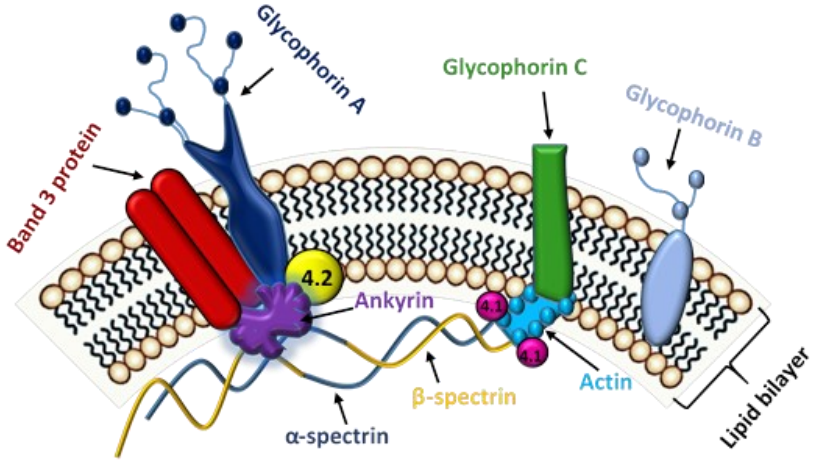

Figure 2. Simplified schematic representation of the red blood cell membrane composition

More details regarding the red blood cell membrane components in Figure 2 and their specific functions are presented in Table 1.

Table 1. Details regarding the major components of RBC membrane

Type	Component	Role and details	Ref
Proteins	Band 3 (AE1/ SLC4A1/ capnophorin)	- **transmembrane protein anchoring the membrane skeleton** **- Anion transporter** - the most abundant protein in membrane consisting of 911 amino acids	[16,17]
	Glycophorins A, B	- are encoded by 2 genes that are similar (GYPA, GYPB) **- carry the MN and Ss blood groups** - glycophorin A is the most abundant	[18,19]
	Glycophorins C, D	- are encoded by a similar gene (GYPC) **- glycophorin C carries the Gerbich blood group system** **- glycophorin C aids in maintaining the shape of RBC** - glycophorin C is specific to humans - glycophorin D is conserved for all apes	[18,20]
	Spectrin α and β	**- cytoskeletal proteins maintaining the shape and flexibility of the membrane** **- spectrin can affect reological properties of blood** - there are 2 α and 5 β spectrin subunits - α and β spectrin subunits mainly associate through hydrophobic bonds supported by electrostatic forces	[21-23]
	Actin	**- short actin filaments (approx. 37 nm in length) connect with spectrin tetramers forming RBC skeleton** **- control the mechanics of RBC**	[24-26]

	Ankyrin	- cytoskeletal protein **- anchors the cytoskeleton to the RBC membrane** **- attaches band 3 and protein 4.2 to β spectrin and skeleton** - Ankyrin's compression into a spring-like state may be a factor in the distinctive shape and flexibility of RBC cells.	[27,28]
	4.2	- peripheral membrane protein - 5% of membrane content - its absence leads to hereditary spherocytosis **- binds to the N-terminal cytoplasmic domain of band 3** **- interacts with ankyrin**	[29,30]
	4.1	**- forms a ternary complex with spectrin and actin** - the attachment to glycophorin C bridges the protein network to the bilayer	[31]
Lipids	Phospholipids	- arranged in an asymmetrical manner into a bilayer with a thickness of two molecules - extracellular surface is more abundant in phosphatidylcholine with sphingomyelins and a small amount of phosphatidylserine - the inner part of the bilayer is mostly abundant in amino phospholipids, primarily consisting of phosphatidylethanolamines, phosphatidylserines and phosphatidylcholine with smaller levels of sphingomyelins **- aid with the fluidity of RBC**	[32,33]
	Neutral lipids	- comprised of mostly cholesterol that is intercalated in between the phospholipid molecules **- aid with the fluidity of RBC**	[32]

As mentioned previously, carbohydrates form about 8% of the dry mass of the erythrocyte membrane. They are mostly relevant for researchers as part of glycoproteins, glycopeptides or glycolipids. The carb residues bound to glycophorins for example can be better observed in Figure 2. Within the red blood cell membrane, carbohydrates are present in many structures such as the Lewis, MN and ABO blood-group specific antigens [34]. The ABO blood grouping, for example, is conditioned by differences in glycoproteins (protein carbohydrate complexes). Type A blood has type A glycoprotein, type B blood has type B glycoproteins, type AB is characterized by the presence of both and type O by the presence of none.

Again, while red blood cells are very complex and many intricacies will not be discussed here, aquaporins must be mentioned. They were first discovered in human RBCs [35-39] and later purified [40] and further studied in relation to their water transport property [41, 42]. Aquaporins or water channel proteins have a primary function of transporting water across cell membranes as a response to

osmotic gradients. The presence of these proteins is integral for RBCs and their proper functioning.

2.1. Transfusions and further prospects

Erythrocytes have a life span of around 120 days before being removed by the spleen. This dramatically changes however when it comes to shelf life. Blood in blood banks, typically stored at 4 °C, is viable for up to 42 days. Of course, during storage some cells might undergo hemolysis at a swifter pace than others, releasing hemoglobin (Hb) in the bag or once they have undergone transfusion [43]. Transfusion of RBCs at the end of their shelf life is unadvisable as it can lead to an increased risk in mortality [44]. This poses a conundrum for patients suffering from trauma-related severe blood loss or different types of blood conditions requiring periodic transfusions. This is a particular concern when it comes to O negative blood types. Cryopreservation would be one solution to such problems.

Erythrocytes preserved in 40-percent (weight/volume) glycerol are approved for storage at -80 °C up to 10 years by the USA-based Federal Drug Administration (FDA) [45]. According to Chang et al. the FDA consents to the employment of such RBCs (processed in ACP 215 Automated Cell Processor (Haemonetics Corp., Baintree, MA, USA)) for only up to 2 weeks since the removal of glycerol [46]. According to a double-blind randomized trial [47], cryopreserved red blood cells (CPRBC) are superior to liquid preserved ones (LPRBC), leading to the absence of an inflammatory response, having an attenuated fibrinolytic state as well as an increased level of 2,3-DPG. Here, the 2,3-DPG (2,3-diphosphoglycerate) is a crucial parameter that influences the proficiency by which hemoglobin releases oxygen to the tissues.

However, while hemolysis is an unwanted factor when it comes to preserving human life, it can have alternative uses. The low shelf life and degradation of RBCs outside of the human body can be used in forensic science towards determining the time at which a certain criminal act was committed. The characteristics of erythrocytes undergo degradation from the moment they encounter the environmental conditions outside of the human body. Hemoglobin for example, is present in 2 forms within the body (with and without oxygen) and while outside of the body it becomes completely saturated with oxygen, auto-oxidizes to methemoglobin, and then suffers a denaturation process to hemichrome [48].

The autoxidation of hemoglobin was reported to comprise an initial fast decay followed by a slower one [49]. One study [50] researched the use of a non-destructive method, namely Attenuated Total Reflection – Fourier Transform Infrared (ATR-

FTIR) spectroscopy, for bloodstain identification in mimicked crime scenarios. This however proved more applicable for older stains, particularly 7-85 days.

The physical characteristics, namely morphology and elasticity, can also be analyzed in comparison to those of the standard, fresh erythrocyte, to determine the time at which a certain crime had taken place. Numerous analysis techniques have been employed to determine the age of a certain bloodstain. One such technology is hyperspectral imaging (HSI), which is favored because it is less invasive, although it is not without drawbacks. Edelman et al. [51] simulated a crime scene with blood stains of various ages (0.1; 2; 15; 40 and 200 days) and measured the temporal behavior of certain blood fractions such as oxyhemoglobin (HbO_2), methemoglobin (MetHb) and hemichrome (HC). This study reports that a mean difference between the actual age of the stain and the measured one is placed in the 0.9-6-day range.

This discrepancy has been extensively explored in the forensic scientific literature, and various analysis approaches have been tested. High resolution electron microscopies might offer an insight into the visible morphological characteristics of red blood cells. Nonetheless, this might not always lead to enough information. However, Atomic Force Microcopy (AFM) can provide an advantage as it not only allows for morphological studies through simple imaging, but it can also give insight into the viscoelastic properties of those RBCs through force measurements. One specific study [52] observed the changes in erythrocytes over a span of 4 weeks by Atomic Force Microcopy. Here, while morphology was not necessarily altered, the elasticity parameters of RBC did decrease over time, a change attributed to drying and coagulation. Another study [53] working with blood from donors (4 females and 4 males; 20-40 years old) managed to identify statistically quantifiable changes in some morphological characteristics of erythrocytes, namely cell perimeter, height, area, and volume during a 28-day experiment.

A decrease in elasticity after 21 days could probably be attributed to an alteration of lipid conformation within the membrane. It was determined that the substrate used for sample deposition is an important factor in these types of time-dependent analyses. An additional information to consider is that the indentation process is a crucial aspect of accurately determining RBCs Young Modulus parameters by AFM. As reported by Smijs et al. [54] the material from which the used cantilever is made does influence the measurements, especially as the stiffness of erythrocytes increases over time.

3. Erythrocyte abnormalities in terms of morphology

Erythrocytes are formed from stem cells found in bone marrow. To reiterate, they appear as biconcave disks, possessing no organelles, but containing hemoglobin. This hemoglobin is however homogenously distributed towards the peripheries of red blood cells, leaving a central pallor area (30-45% of RBC

diameter) [55]. Also, the normal erythrocyte membrane appears to be smooth without any protuberances and is basically indistinct from the Hb content. There are some factors that can alter the morphology of normal RBCs such as an alteration (either increase or decrease) in Hb volume; a change in Hb structure (i.e. Hb variant, HbC, sickle cell Hb; protective enzyme deficiency leading to polymerization and precipitation); increase level of erythrocyte destruction or increase in level of demand (i.e. insufficient hemoglobinization of cells; absence or poorly functioning spleen) [56].

RBCs must maintain structural characteristics and a high amount of flexibility in order to function properly. However, a variety of abnormalities linked to certain pathologies have been described in literature and medical practice, as they were observed on peripheral blood smears. Table 2 presents, in alphabetical order, all existent such pathogenic abnormalities of RBCs (to the author's knowledge and based on available research) for simplicity of access.

Table 2. RBC pathologic variations

Name	Description	Ref.
Acanthocyte (spur cell)	Described as having long projections that are irregular (spicules). They are also characterized as having no central pallor. It is mainly associated with severe liver disease, partial or total removal of the spleen and some rare cases of abetalipoproteinemia or neuroacanthocytosis (McLeod and choreo-acanthocytosis syndrome).	[57,58]
Anisocytosis	The sizes of erythrocytes can vary within a wide range	[59-61]
Babesiosis	This appears similar to the ring forms in malaria	[62,63]
Basophilic stippling	Red blood cells present small RNA aggregates which are seen as little blue dots. In this case, fewer dots can be a characteristic of reticulocytes and more dots that of toxic bone marrow damage, thalassemia or myelodysplasia	[64,65]
Elliptocyte	The cell appears elongated and elliptical and it is associated with rare hereditary elliptocytosis	[66,67]
Echinocyte	Similar to acanthocytes but the spicules or projections are regular. Might be associated with uremia/renal disease	[58]
Heinz type body	Precipitated hemoglobin is visible after supravital staining as a perimembranous blue dot, common with hemoglobinopathies	[68,69]
Howell-Jolly type body	Commonly seen with the absence of the spleen, observed as a deeply basophilic nuclear remainder that is small and round	[70,71]
Hypochromic	Presented as cells with an increased mean corpuscular hemoglobin (MCH) and it is specific to a deficiency in iron. A RBC is considered hypochromic if the central pallor is measured at more than 50 % of the RBC diameter.	[72,73]
Macrocytosis	Red blood cells present an increased mean corpuscular volume (MCV) and it is typically associated with megaloblastic anemias. Macrocytes measure typically more than 9 μm in diameter	[74,75]

Malaria	Infections with the single-cell parasite that causes malaria can be observed as ring-forms, dots or gametocytes	[76-78]
Microcytosis	Red blood cells present a decreased mean corpuscular volume (MCV) and it is linked to iron deficiencies and thalassemia types. Microcytes measure on average less than 6 μm in diameter.	[79,80]
Pappenheimer type body	Described by the presence of little blue dots that contain iron. This is typically linked to an iron overload or to the absence of the spleen	[81,82]
Poikilocytosis	Red blood cells vary in shapes	[83]
Polychromatophilia	Young erythrocytes present a bluish tint due to high a content in RNA	[84]
Reticulocyte	Supravital staining can precipitate young RBCs with higher RNA content for identification and counting	[85,86]
Rouleaux	More serum protein, especially more fibrinogen or globulin, reduces surface charge and causes a linear aggregation of RBCs that resembles a stack of coins.	[87,88]
Sideroblast	Non-nucleated erythrocytes that have stainable iron	[89,90]
Spherocyte	RBCs appear smaller, rounder, and denser without any presence of a central pallor. This has been linked to splenic hemolysis in normal patients. In patients suffering from hereditary spherocytosis and increase in osmotic fragility can be seen	[91,92]
Schistocyte (schizocyte)	RBC shapes are presented as irregular and fragmented, and this is mainly associated with intravascular hemolysis (i.e. microangiopathic hemolytic anemias). Alternative that appears cut in half (i.e. helmet cell) or with 2, 3 pointed projections on the surface (i.e. horn cells/ keratocytes) also exist.	[93,94]
Sickle cell (drepanocyte)	Red blood cells in sickle cell pathologies are seen as curved-shaped, with the ends being pointed. This is due to aggregation of hemoglobin S	[95,96]
Stomatocyte	Erythrocytes with a central pallor that is similar to a slit, sporadically non-specific or artefactual stomatocytes. This is linked to rare hereditary stomatocytosis	[97,98]
Target cell (codocyte)	Cell with central and peripheral staining and an intervening pallor from increased RBC membrane redundancy. This can be observed in the case of some thalassemia types, liver disease, as well as hemoglobin C disease	[58, 99]
Tear drop cell (dacrocyte)	Red blood cells are pinched at one end, hence the name. These specific types of cells are linked to myelophthisic conditions and myelofibrosis	[100]
Wafer cell (leptocyte)	The hemoglobin is present at the periphery of cells which are thin and flat in appearance. Leptocytes tend to fold into bowl-shaped cells	[101]

While erythrocytes are essential for human life, due to their role in transporting oxygen, they have generally been considered inert from an immunological point of view. However, it was recently shown that they also

function as immunological sensors due to the surface expression of the nucleic acid-sensing toll-like receptor (TLR9). According to Hotz et al. erythrocytes can bind mitochondrial DNA to prevent lung injury [102]. This intrinsic immune response can act in a favorable way in the removal of damaged erythrocytes. Moreover, it also plays a part in systemic inflammation as well as the progression of anemia in pathologic states with elevated free-cell DNA.

Red blood cells are also capable of binding pathogenic organisms, the most notable of which being the *Plasmodium* species, which is associated to malaria. It is reported that different species within this genus have distinct mechanisms for adhering to RBCs [103]. For example, the most virulent malaria pathogen (*P. falciparum*) adheres to glycophorins (A, B or C) and therefore can invade RBCs [104] to multiply inside and thus evade any repercussions from the immune system. This invasion leads to changes in erythrocytes, most notably a loss of normal shape, increased permeability to ionic species and an increased rigidity of the membrane [105].

In a recent study by Lam et al. [106] it was shown that when compared to healthy erythrocytes which have only a small percentage of TLR9 on their membrane, those from malaria patients have up to 40%. Because RBCs lack a nucleus to regulate gene expression in response to environmental factors, such changes in TLR9 protein were explained by DNA testing as being linked to changes in the membrane and shape in order to expose more of this protein. In addition, in light of the ongoing global health issue, it was discovered that the SARS-COV-2 virus can infect RBC precursors [107]. Therefore, erythrocyte production (erythropoiesis) under stress is part of the response to this virus. According to Marchi et al. [108] certain morphologies related with the infection rather than any comorbidities have also been recorded for COVID-19 individuals. Most patients in this study had spiculated cells, which could be an indicator of erythrocytes with impaired protein and lipid membrane composition.

4. RBCs and their interaction with drugs

Most drugs have potential side effects especially at higher doses or when used over extended periods of time. It is crucial to obtain the maximum therapeutic result with as few repercussions as possible. Drug activity and potency, on the other hand, are affected by a variety of parameters such as adsorption, distribution, and metabolism. Drugs are transported within the blood stream either in bound form (e.g. RBCs, plasma proteins) or in unbound form. Only the unbound form can be available for passive diffusion to sites, and typically this determines drug efficacy. Thus, the interactions of drugs with cell membranes is important to understand. However, due to the complexity of natural cell membranes, several biomimetic models have been proposed throughout the years,

for example, monolayers [109-118], bilayers [119-121] or Langmuir-Blodget layers [122-128]. While key towards understanding interactions with drugs at a molecular level, they present certain drawbacks. Here, probably the most important one is the challenging aspect of incorporating proteins in such models, a conundrum since membrane proteins tend to have an impact on the adsorption, distribution, metabolism, and excretion of drugs. Nevertheless, such models offer valuable insights albeit their individual pros and cons.

Even so, the specific interaction of erythrocytes with intravenous injectable-type drugs (e.g. antibiotics, chemotherapy agents or insulin) is particularly important as they do interact with each other intravenously. Each drug has a different affinity to RBCs, and it is reported that there is a battle between erythrocytes and blood plasma proteins for the free drug present within the bloodstream [129]. Plasma protein binding (mostly serum albumin and α1-acid glycoprotein) is unwanted as it can take away from drug potency [130]. Serum albumin binds mostly acidic drugs while more basic drugs are bound to lipoproteins, alpha-1 acid glycoprotein, or both [131].

In theory, the more lipophilic a medicine is, the more it binds to red blood cells; non-lipophilic substances are not dispersed to RBCs at all. Many lipophilic compounds will dissolve in RBCs lipid bilayer and then penetrate the cell. However, there are highly lipophilic substances that have been reported to have a higher protein binding affinity and thus do not necessarily bind well to RBCs. One such example is tetrahydrocannabinol that binds well (96-98%) to washed out erythrocytes (in the absence of plasma proteins) but very poorly (7-9%) to RBCs in whole blood (in the presence of plasma proteins) [132]. Substances that possess a high affinity to erythrocytes have a large blood to plasma ratio (R_b), where:

$$R_b = \frac{blood\ concentration\ of\ drug}{plasma\ concentration\ of\ drug} \quad [133]$$

The scientific literature offers substantial information on the effects of various chemicals on RBCs, with some recent research focused on changes in erythrocytes caused by drug binding. For example, Zdrenghea et al. [134] investigated the effect of procaine ($5*10^{-7}$ M, $5*10^{-5}$ M, $5*10^{-4}$ M) on a human erythrocyte membrane revealing changes at the micro and nanometer scales.

The aggregation of membranous particles on the cell surface and the creation of domain structures brought on by procaine are two factors that can be linked to changes in the surface morphology of the erythrocyte membrane. One other study [135] presents findings, through X-ray diffraction, on the effect of aspirin (1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM) on the structure of RBC membranes. While the concentration of aspirin had no effect on the size of the lipid domains, there was a little increase (30 to 38 Å) in the size of the peptide domain. According to the

authors, membrane thickness is lowered while the distance between lipid tails increases, indicating membrane fluidification.

In erythrocytes, it is reported that there are 3 major components that have the capability to bind drugs, namely hemoglobin, carbonic anhydrase (intracellular enzyme that catalyzes the $CO_2 + H_2O \rightleftarrows H_2CO_3$ reaction and is found in RBC in high concentrations) and the actual cell membrane [129].

One study [139] found that Penicillin G, dicloxacillin, tetracycline and minocycline tend to bind not only to hemoglobin but to the globin moiety, in the absence of iron, after the dissociation of heme. Out of the four antibiotics, tetracycline stands out as it can also bind to carbonic anhydrase with zinc being required for the binding. More recent molecular docking studies [137] confirmed that certain drugs have an affinity for the specific protein moiety of hemoglobin – in this case, a lipid-lowering agent (bezafibrate) and a diuretic used for blood pressure (hydrochlorothiazide).

Another two diuretics, namely chlorthalidone and acetazolamide have been proven to bind to carbonic anhydrase. Both compete for the same binding sites and acetazolamide tends to inhibit the uptake of chlorthalidone throughout mixed treatments and being able to displace it at the binding sites [138]. Also, it was demonstrated that the accumulation process of acetazolamide within RBCs is composed of two parts, namely a non-linear one that is saturable and a linear one which is controlled by diffusion [139].

As for the RBC membrane and its interaction with drugs, for example, Bickel et al. [140] investigated the binding of an anesthetic (chlorpromazine) a tricyclic antidepressant (imipramine) and an anti-inflammatory agent (salicylic acid). It was discovered that chlorpromazine mainly binds to the membrane, although there is a possible interference as hemoglobin tends to catalyze the formation of chlorpromazine-sulphoxide. Imipramine has quite a negligible binding to erythrocyte contents, being bound mainly to its membrane. Salicylic acid on the other hand has little affinity for red blood cells, being quite partial to plasma proteins.

The examples discussed above show that drugs can behave differently, even if they are part of the same class. A better understanding of such interactions is of particular importance considering the emerging research trend of using RBCs as drug or nanoparticle delivery vehicles. Given their biocompatibility and long-term free circulation throughout the circulatory system, this is extremely possible.

These would undoubtedly extend the drug's life, which is critical for medicine that has a gradual release in blood or has a purpose of action within the intravascular area within places accessible to erythrocytes [141]. The ability to help drugs bypass metabolism mechanisms also make RBCs the ideal choice for drug delivery.

The incorporation of pharmaceuticals into red blood cells will not only guard against immune cell phagocytosis but also prevent any potential biotoxicity from the drug itself [142]. Conversely, an alternative to encapsulation is that of coupling drugs onto the erythrocyte membrane. While a concern can be raised regarding drug isolation, this is not valid when it comes to pharmaceuticals that are supposed to be active within the bloodstream [143].

5. Other considerations

While the need to study the "classic type" of injectable drugs in relation to blood components and specifically red blood cells is quite obvious, concerns can be raised in regard to other substances/devices especially pertaining to implantology. This applies even to biocompatible and bioactive types of resorbable materials. One such example would be hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAP) a substance widely investigated for bone substitutes due to its similarity to the mineral component in human bone tissue [144-163].

Even though HAP-based implants (as a coating on metallic implants or in composites) are used at bone sites and they are technically biocompatible and bioactive, aiding in bone proliferation, there are certain things to consider. Ions (i.e. calcium and phosphate for unsubstituted HAP) can leach into the bloodstream. This can be somewhat problematic, depending on several factors such as size and type of implant or different pathologies of the patient in cause.

Intracellular calcium ions are a key factor in erythrocytes, influencing aspects including metabolic activity, redox state, and cell clearance in addition to biophysical characteristics (i.e. membrane composition, volume, and rheological characteristics) [164]. Ca^{2+} can cause K^+ efflux (Gardos effect) after it enters RBCs, causing cell degeneration (e.g., loss of intracellular fluid and volume decrease) [165,166]. However, the biomedical applications of HAP go far beyond bone implants. Due to their high biocompatibility and ability to be functionalized with different drugs and molecules, HAP nanoparticles have been the subject of a variety of targeted delivery studies.

In this case, the importance of blood compatibility studies and a deeper understanding of how these functionalized nanoparticles would interact with red blood cells and other blood components is especially important. Particulate matter within the bloodstream can hinder the homeostatic balance [167].

Even though this is not a well-investigated topic, there are some studies concentrating on hydroxyapatite and its potential impacts on red blood cells. Table 3 contains examples of some articles discovered by the authors.

Table 3. Hydroxyapatite-based materials and their effects on red blood cells

Type of HAP material	Type of study on RBCs	Effect	Ref.
HAP nanoparticles	*In vitro* 5% centrifuged RBC suspension in 0.9% NaCl solution	- aggregation and adherence to membrane - higher adsorption capacity leads to "caves" on RBC membrane - no rupture in lipid bilayer - no hemolysis	[168]
HAP nanoparticles modified with heparin		- inhibited aggregation - no hemolysis	
HAP microparticles		- no observed adhering to membrane - no hemolysis	
Hydroxyapatite - Polymethyl Methacrylate Nanocomposites (10% HAP)	*In vitro* Blood samples (healthy donors 20-28 years) were mixed with 50% (mg/dL) HAP-PMMA	- decreased RBC lifetime - change in RBC shape and size - lower hemoglobin in 40% samples	[169]
Commercial Rod-shaped HAP (width -15–30 nm, length - 40–70 nm)	*In vitro* Blood - healthy non-smoking adult volunteers Free from medication	~ 0.25% hemolysis at 1 and 10 mg/mL	[170]
Commercial Needle-shaped HAP (width - 30–60 nm, length - 200–500 nm)			
Synthetic HAP diameter: 3-11 nm length: 20-65 nm	RBC centrifuged from BALB/c mice blood 5% (v/v) RBC in saline	- lower hemolytic activity (<5%) compared to control - this may be cause by may be caused by surface hydrophilic groups (carboxyl, hydroxyl functional groups)	[171]
hydroxyapatite magnetic nanoparticles doped with different ions (Gd^{3+}/ Fe^{2+}/ Fe^{3+}/ Co^{2+})	Blood from 4 volunteers	- no hemolytic activity up to concentrations of 4 mg/mL - nanoparticles do not affect the membrane integrity or morphology of RBCs	[172]

6. Conclusions

Recent studies regarding red blood cells have revealed that their functions go beyond that of oxygen transporters, as they play a key role in redox regulation, metabolism control and interorgan communication. However, it is possible to state that they are still understudied. Because erythrocytes are essential for life, more research is needed to understand every aspect of erythrocytes, from their development in the bone marrow to their end of life and various diseases, to move forward with different clinical applications.

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