

Review

Classical Effective Techniques to Evaluate Biological Compounds and Materials Toxicity Using Red Blood Cells as Biosensors

César Iván Romo-Sáenz ^{1,2} , Nancy Edith Rodríguez-Garza ^{1,3,*} , Ana Laura Delgado-Miranda ¹,
Diana Laura Clark-Perez ¹ , Beatriz Elena Castro-Valenzuela ⁴, Celia María Quiñones-Flores ²,
Alva Rocío Castillo-González ², Andrés García ⁵ , Patricia Tamez-Guerra ¹  and Ricardo Gomez-Flores ^{1,*}

¹ Departamento de Microbiología e Inmunología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolas de los Garza 66455, Nuevo León, Mexico; cromo@uach.mx (C.I.R.-S.); laura.delgadam@uanl.edu.mx (A.L.D.-M.); diana.clarkp@uanl.edu.mx (D.L.C.-P.); patricia.tamezgr@uanl.edu.mx (P.T.-G.)

² Facultad de Medicina y Ciencias Biomédicas, Universidad Autónoma de Chihuahua, Chihuahua 31125, Chihuahua, Mexico; cquinonezf@uach.mx (C.M.Q.-F.); acastillo@uach.mx (A.R.C.-G.)

³ Campus Monterrey, Universidad Tecnológica de México (UNITEC), San Nicolas de los Garza 66499, Nuevo León, Mexico

⁴ Facultad de Zootecnia y Ecología, Universidad Autónoma de Chihuahua, Chihuahua 31453, Chihuahua, Mexico; bcastro@uach.mx

⁵ Laboratorio de Biotecnología Ambiental, Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Cuernavaca 62209, Morelos, Mexico; andres.garcia@uaem.edu.mx

* Correspondence: nancy.rodriguezgr@uanl.edu.mx (N.E.R.-G.); ricardo.gomezfl@uanl.edu.mx (R.G.-F.)

Abstract

Red blood cells represent a widely used cellular model in cytotoxicity studies, particularly in hemocompatibility assessments. As enucleated cells, which are abundant and easily accessible in both humans and animals, red blood cells allow for rapid, reproducible, and low-cost evaluation of the toxicity of bioactive compounds, whether natural, synthetic, or nanoparticulate. From a functional perspective, the red blood cell membrane is highly sensitive to physical and chemical environmental changes (osmolarity, temperature, pH, and the presence of oxidizing agents). This sensitivity makes red blood cells an effective biosensor for detecting membrane damage, hemolysis, oxidative stress, methemoglobin formation, and aggregation processes. Therefore, in vitro tests using red blood cells allow for the preliminary evaluation in preclinical development, particularly for the early screening of cytotoxicity, membrane-disruptive effects, and hemocompatibility of small molecules, nanomaterials, and blood-contacting biomaterials. These techniques include hemocompatibility tests, evaluation of oxidative and osmotic damage, and evaluation of erythrocyte aggregation and function. However, the use of red blood cells as a cytotoxicity model also has significant limitations. As anucleate cells, erythrocytes lack organelles such as nuclei, mitochondria, or lysosomes, which prevents the evaluation of their effects on key intracellular processes such as protein synthesis, cell signaling, apoptosis, or endoplasmic reticulum stress. This lack of cellular complexity limits their usefulness as a sole model in studies of systemic toxicity or tissue-specific cytotoxicity. These tools offer an effective preliminary approach to anticipating risks in biomedical and pharmacological research.

Keywords: red blood cells; cytotoxicity; biocompatibility; hemotoxicity; hemocompatibility



Received: 26 January 2026

Revised: 22 February 2026

Accepted: 25 February 2026

Published: 2 March 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

The ongoing development of drugs, nanoparticles, natural formulations, medical devices, and other therapeutic agents is one of the main strategies for disease control and

treatment worldwide [1,2]. However, factors such as the growing resistance to conventional treatments, biological variability, and the limited availability of effective medications require the design of new therapeutic alternatives that are not only effective but also safe for the body [3].

Despite the therapeutic potential offered by various natural and synthetic sources, it is essential to evaluate their biocompatibility, especially in compounds that interact with the circulatory system [4]. In this context, compatibility with erythrocytes becomes particularly relevant, since these abundant, enucleated cells are highly sensitive to physical and chemical effects [5]. Exposure to bioactive compounds induces hemolysis, hemoprotection, membrane oxidation, osmotic fragility, or abnormal aggregation, which may compromise erythrocyte functionality and trigger systemic adverse effects [6].

Various *in vitro* and *in vivo* tests have been implemented to evaluate the compatibility of organic and inorganic compounds and even medical devices, in order to minimize future side effects, when implemented in humans or animals [2,7]. However, many of the current biocompatible evaluation techniques require sophisticated infrastructure or involve high costs, which hinders their routine use in laboratories with limited resources [8].

The evaluation of biocompatibility, as well as the selective toxicity of nanostructured compounds, biomaterials, and bioactive compounds, represents a critical stage during the early phases of pharmacological development with biomedical impact. Initially, the interaction of these materials with easily accessible and rapidly obtainable biological models, such as erythrocytes, is particularly relevant, as red blood cells are highly sensitive to physicochemical alterations resulting from direct contact with tested compounds. Therefore, erythrocytes have been widely employed as a simple, accessible, and physiologically relevant model for the preliminary screening of compound selectivity and toxicity, including nanocomposites [9,10].

In recent years, several studies have demonstrated the importance of erythrocytes as cytotoxicity models, highlighting their role in the development of biosensors for membrane damage, oxidative imbalance, and hemolytic activity. Nevertheless, most of these studies focus on specific classes of materials, such as nanoparticles, implantable biomaterials, or molecular mechanisms, rather than providing a comprehensive methodological perspective of the diverse assays that can be integrally implemented in a preliminary analytical workflow [11–13].

Despite the widespread use of classical assays, such as hemolysis, oxidative damage, osmotic fragility, erythrocyte aggregation, and methemoglobin formation, experimental variations, interpretation thresholds, and reporting criteria across different studies continue to represent a major comparative challenge. This lack of methodological standardization has been recognized as an important limitation for inter-study comparison and result reproducibility [14–16]. In parallel, recent reviews focusing on antioxidant activity or assay optimization emphasize the continued relevance of erythrocyte-based models [17].

In this scenario, classical hemocompatibility techniques based on human or animal erythrocytes remain highly accessible, sensitive, and reproducible tools [11,18]. These tests allow for the early identification of potential toxic effects and may serve as, or be considered as, an effective, economically reproducible, and low-cost preliminary step before advancing to more complex studies.

Therefore, the present review was considered necessary to consolidate, update, and critically analyze the classical erythrocyte-based methods that are currently employed to evaluate the toxicity and hemocompatibility of biological compounds and materials. Unlike previous reviews, this work integrates the experimental basis, biological relevance, advantages, and limitations of each assay, while also proposing practical interpretation criteria to facilitate comparison across studies. In doing so, this review aims to provide researchers

with a comprehensive and practical reference for the rational selection of erythrocyte-based assays as a preliminary screening tool prior to advancing to more complex in vitro or in vivo models.

2. Erythrocyte Hemocompatibility

2.1. Biophysical Properties of the Erythrocyte Membrane

The erythrocyte membrane is crucial for erythrocyte compatibility, as it is the barrier that separates intracellular content from the extracellular environment. Its structure and biophysical properties largely determine the response of erythrocytes to exposure to compounds [10]. The lipid membrane, composed mainly of phospholipids and cholesterol, is involved in selective permeability and in maintaining the biconcave shape of erythrocytes, which is essential for their flexibility and functionality [19].

2.2. Structure and Properties of the Erythrocyte Membrane

The red blood cell membrane is organized into a lipid bilayer that is anchored to an underlying protein network known as the cytoskeleton, composed primarily of spectrin and actin [20]. This structure gives red blood cells their flexibility, allowing them to navigate through the narrowest capillaries and preventing their rupture [21]. The membrane also contains several integral proteins, such as the anion channel, the chloride–bicarbonate transporter, and various glycoproteins, which are essential for pH regulation and cell integrity [22,23].

2.3. Interactions with External Compounds

Exposure of erythrocytes to bioactive compounds can induce significant alterations in membrane stability and cellular homeostasis. These interactions may promote oxidative stress through the excessive generation of reactive oxygen species (ROS), which play a central role in erythrocyte damage. Due to their high polyunsaturated fatty acid content, erythrocyte membranes are particularly susceptible to oxidative attack, making them a sensitive model for toxicological assessment. ROS can initiate lipid peroxidation processes by targeting bilayer phospholipids, as well as oxidizing cytoskeletal proteins and hemoglobin, leading to structural and functional impairment of the erythrocyte membrane [24]. Oxidative modification of membrane proteins and lipids disrupts membrane fluidity, deformability, and permeability, which compromises the mechanical stability of erythrocytes during circulation. As a consequence, these alterations can increase membrane fragility, ultimately resulting in hemolysis and the subsequent release of hemoglobin into the plasma [25]. Therefore, erythrocyte-based assays provide a valuable and sensitive approach for evaluating the cytotoxic and pro-oxidant effects of bioactive compounds and biomaterials, as well as their potential to induce membrane damage under physiological conditions.

On the other hand, exposure of erythrocytes to bioactive compounds can induce membrane damage through multiple pathways, which largely depend on the chemical structure, physicochemical properties, and redox behavior of the compound. Different classes of bioactive compounds trigger membrane injury through distinct mechanisms, including direct interaction with the lipid bilayer, metal-catalyzed redox reactions, hemoglobin modification, or disruption of cytoskeletal proteins [9,10].

Amphiphilic and surface-active compounds, such as surfactants, saponins, and antimicrobial peptides, interact with erythrocytes primarily through direct insertion into the lipid bilayer. Their amphipathic structure facilitates partitioning into membrane lipids, thereby altering lipid packing, membrane fluidity, and surface tension. This interaction promotes pore formation and osmotic lysis, processes that frequently occur independently

of oxidative stress. The hemolytic activity of these compounds correlates with hydrophobic chain length, charge density, and overall amphiphilic balance [26,27].

Metal ions and metal-based nanoparticles induce erythrocyte damage predominantly via catalytic redox mechanisms. Transition metals such as iron, copper, and silver promote Fenton or Fenton-like reactions, generating highly reactive hydroxyl radicals at the membrane surface. These reactive species initiate localized lipid peroxidation and protein oxidation, making membrane damage more dependent on oxidation state, coordination chemistry, and surface reactivity than on strictly dose-dependent toxicity [12,13].

Redox-active organic compounds, including quinones and related conjugated systems, exert their toxicity through redox cycling mechanisms. These compounds undergo repeated reduction–oxidation reactions, resulting in sustained generation of reactive oxygen species and rapid depletion of erythrocyte antioxidant defenses. Structural features such as conjugated ring systems, electron-withdrawing substituents, and redox potential determine their pro-oxidant efficiency and the extent of both membrane and hemoglobin oxidation [28,29].

Hemoglobin-reactive agents, such as phenylhydrazines and nitrite-derived compounds, primarily target intracellular hemoglobin rather than membrane lipids. These substances induce hemoglobin denaturation, methemoglobin formation, and Heinz body aggregation. Secondary interactions between damaged hemoglobin and cytoskeletal proteins reduce erythrocyte deformability and promote premature hemolysis, with membrane rupture occurring as a downstream consequence of intracellular protein damage [30,31].

Polyene antibiotics and other sterol-binding agents interact selectively with membrane sterols, forming transmembrane pores that disrupt ionic balance and membrane integrity. Their hemolytic activity depends on structural complementarity between the polyene macrolide backbone and cholesterol, underscoring the importance of lipid composition and molecular recognition in erythrocyte susceptibility [12].

Finally, compounds that target the cytoskeleton compromise erythrocyte integrity by modifying spectrin–actin interactions or associated anchoring proteins. Oxidative modification or covalent binding to cytoskeletal components reduces membrane elasticity and mechanical stability, increasing sensitivity to shear stress and promoting mechanical hemolysis during circulation [32,33].

2.4. Oxidative Stress and Activation of Cellular Pathways

Oxidative stress is one of the most common molecular responses in erythrocytes exposed to potentially toxic compounds. When erythrocytes are exposed to an environment with a high concentration of reactive oxygen species (ROS), endogenous antioxidants such as glutathione peroxidase and superoxide dismutase are involved in neutralizing these species [34,35]. However, if the amount of ROS exceeds the antioxidant activity, lipoperoxidation of membrane lipids may occur, leading to alterations in the fluidity and stability of the lipid bilayer, and ultimately, cell lysis [36].

In addition, some compounds induce the activation of immunological pathways, such as complement activation, which is triggered by the formation of immune complexes on the surface of erythrocytes, promoting opsonization and facilitating phagocytosis [37,38]. Although this activation is more relevant in the context of extrinsic materials, certain drugs or nanoparticles also induce adverse immunological reactions [39,40].

In addition to oxidative damage, some compounds induce erythrocyte injury through activation of immunological pathways, particularly the complement system. Complement activation may be triggered by antibody–antigen complexes or foreign surfaces interacting with erythrocyte membrane proteins, leading to opsonization and formation of the membrane attack complex (MAC), which can cause intravascular hemolysis or promote extravascular clearance by phagocytes [41,42].

Another important pathway involves dysregulation of intracellular calcium (Ca^{2+}) homeostasis. Oxidative stress or membrane-interacting compounds can increase Ca^{2+} permeability, resulting in elevated intracellular Ca^{2+} . This influx activates Ca^{2+} -dependent enzymes and disturbs membrane phospholipid asymmetry, ultimately reducing erythrocyte deformability and increasing susceptibility to damage. Elevation of intracellular Ca^{2+} is also a major driver of eryptosis, a form of programmed erythrocyte death characterized by cell shrinkage, membrane blebbing, and phosphatidylserine exposure, which promotes macrophage recognition and clearance independent of direct hemolysis [43].

2.5. Erythrocyte Aggregation

Red blood cells aggregate in response to alterations in the physicochemical properties of blood. This may occur through interactions between the glycocalyx of red blood cells, or through changes in the membrane surface charge caused by certain compounds [44]. Abnormal red blood cell aggregations affect blood viscosity and alter microvessel flow, compromising blood circulation and promoting thrombus formation [45].

2.6. Molecular Considerations on Hemocompatibility

The compatibility of a compound with erythrocytes depends on its structural characteristics and its physicochemical properties, which include its charge, size, and shape [13]. Compounds with a negative or neutral charge are usually better tolerated by erythrocytes, whereas compounds with positive charges induce greater electrostatic interaction with the membrane that triggers hemolysis or oxidative stress [46,47]. Similarly, the particle size (in the case of nanoparticles) may influence the potential of the compound to induce erythrocyte aggregation or mechanical hemolysis [12].

3. Critical Aspects and Sources of Variability in Erythrocyte Preparation for Hemocompatibility Assays

Proper red blood cell preparation represents a major source of variability in hemocompatibility assays and directly affects the reliability and interpretation of experimental outcomes [14,48]. The preparation steps summarized below are representative of commonly reported practices and are discussed in terms of their potential influence on assay reproducibility when preparing human or animal red blood cells:

1. **Blood collection:** Fresh blood is obtained from human or animal donors, following ethical and safety regulations. The blood must be treated with an anticoagulant, such as EDTA (ethylenediaminetetraacetic acid) or citrate, to prevent clotting [14].
2. **Centrifugation:** The blood sample is centrifuged at 3000 rpm for 10 min to separate the cellular components from the blood. The plasma is carefully removed, leaving the red blood cells at the bottom of the tube [49].
3. **Red blood cells washing:** Red blood cells are washed three times with isotonic saline, such as 0.9% NaCl or phosphate-buffered saline (PBS), to remove plasma residues. Centrifugation is performed at 3000 rpm for 5 min between each wash [50]. Proper red blood cell preparation represents a major source of variability in hemocompatibility assays and directly affects the reliability and interpretation of experimental outcomes [51].
4. **Red blood cell suspension:** After washing, red blood cells are suspended in an isotonic solution (0.9% NaCl or PBS pH 7.2, depending on the assay) at a concentration of approximately 2% to 5% by volume [50].

Proper red blood cell preparation is a critical factor influencing the reproducibility of hemocompatibility assays and the interpretation of their results. Variations in sample handling, washing procedures, and experimental conditions have been widely reported to

introduce significant variability, potentially leading to inconsistent or misleading outcomes across studies [52].

3.1. Hemocompatibility Tests

Classical hemocompatibility tests are classified according to the type of red blood cell damage being assessed. The parameters for these assays are summarized in Table 1. The tests differ in the mechanism of injury they detect and therefore provide complementary information [53]. Hemolysis-based assays are direct indicators of membrane disruption but do not identify the underlying cause of damage. In contrast, oxidative assays (H_2O_2 exposure, TBARS, and FOX) are more sensitive to early biochemical alterations and allow detection of subhemolytic oxidative stress. Osmotic fragility tests primarily reflect structural membrane stability under physicochemical stress, whereas aggregation assays provide hemorheological information not captured by lysis-based methods. Methemoglobin formation assesses functional impairment of hemoglobin and complements structural endpoints. Together, these approaches vary in sensitivity, specificity, and physiological relevance, supporting their combined use for a comprehensive evaluation of hemocompatibility.

In addition to mechanistic differences, the assays also vary in their experimental parameters. Most hemolysis and oxidative tests share similar incubation temperatures (typically 37 °C) and spectrophotometric detection near 540 nm, enabling comparable quantification of hemoglobin release. However, oxidative assays require broader reaction conditions and, in the case of TBARS, higher temperatures and longer centrifugation steps, reflecting their chemical detection principles. Functional and aggregation tests differ in detection wavelength and handling conditions, indicating that assay selection depends not only on the type of erythrocyte damage assessed but also on analytical sensitivity, processing requirements, and experimental complexity. Collectively, the parameters compiled in Table 1 provide a structured framework for selecting appropriate assays and for comparing methodological requirements across studies.

3.1.1. Assessment of Hemolysis and Membrane Damage

1. Direct hemolysis: Prepared red blood cells are exposed to the compound at various concentrations and incubated at 37 °C for oxidation. The release of hemoglobin into the supernatant is then measured by spectrophotometry at 540 nm. An increase in absorbance indicates hemolysis [54].
2. Heat-induced hemolytic activity: Red blood cells are exposed to specific temperatures (e.g., 50 °C to 60 °C) for 30 min, after which hemolysis is measured through the release of hemoglobin [55].
3. Cold-induced hemolytic activity: Red blood cells are incubated at low temperatures (0 °C to 4 °C) for 30 min, and hemolysis is then measured. This type of test assesses the stability of red blood cells to extreme temperature changes [55].

3.1.2. Evaluation of Oxidative Damage

1. Antihemolytic activity by oxidative damage induced by H_2O_2 : Red blood cells are exposed to various concentrations of H_2O_2 to induce oxidative stress. The compound under study is added to the red blood cells suspension before incubation. After a predetermined time (e.g., 30 min), hemolysis is spectrophotometrically measured. The antioxidant activity of the compound is assessed by its potential to reduce hemolysis [56].
2. Hemoprotection activity by 2,2'-azobis (2-methylpropionamide)-dihydrochloride (AAPH): Red blood cells are incubated with APPH to generate free radicals. The induced hemolysis is then measured and the ability of a compound to protect red blood cells from damage caused by APPH-generated free radicals is assessed [54].

3. Thiobarbituric acid reactive substances (TBARS) test: Erythrocytes are incubated with an oxidizing agent, such as H_2O_2 , to induce lipid peroxidation. Thiobarbituric acid is then added and heated under commonly reported conditions (e.g., $\sim 95^\circ\text{C}$ for 30 min), depending on the specific protocol used. After cooling, absorbance at 532 nm is measured to evaluate lipid peroxidation products, such as malondialdehyde, indicating oxidative damage to erythrocyte membranes [56].
4. Xylenol orange test: Red blood cells are incubated with an oxidizing agent such as H_2O_2 to induce lipid peroxidation. Xylenol orange reagent is then added, which binds to lipid peroxidation products. The mixture is incubated for a specified time, and the absorbance at 540 nm is measured, indicating the level of oxidative damage to the red blood cell membranes [57].

3.1.3. Osmotic Damage Assessment

1. Antihemolytic activity by osmotic damage (hypotonicity): Red blood cells are exposed to NaCl solutions with decreasing concentrations (from 0.9% to 0.1% NaCl). Hemolysis is then spectrophotometrically measured, and the potential of the compound to protect erythrocytes from osmotic hemolysis is assessed [55].

3.1.4. Evaluation of Erythrocyte Aggregation and Function

1. Albumin-induced antiaggregatory activity of erythrocytes: Red blood cells are incubated with albumin at various concentrations to induce aggregation. Red blood cell aggregation is then measured using dark-field microscopy or viscosity techniques, and the potential of the compounds to reduce aggregation is assessed [58].
2. Measurement of methemoglobin in human erythrocytes: Red blood cells are incubated with oxidizing agents to induce methemoglobin formation. The methemoglobin concentration in red blood cells is then measured by spectrophotometry at 630 nm [59].
3. Anti-methemoglobin activity: The potential of a compound to prevent or reverse the formation of methemoglobin is assessed. Red blood cells are exposed to an oxidation source (NaNO_3), and the amount of methemoglobin in the presence of the protective compound is measured [31,59–61].

Collectively, these hemocompatibility assays provide sensitive and early indicators of cytotoxicity and biocompatibility for a wide range of materials, including small molecules, nanomaterials, and blood-contacting biomaterials. Because erythrocytes lack nuclei and repair mechanisms, alterations in membrane integrity, oxidative balance, or deformability directly reflect physicochemical interactions at the cell surface. Consequently, these tests are particularly valuable for identifying sublethal or early-stage damage that may not be detected in more complex cellular models, supporting their use as first-line screening tools in toxicological and biocompatibility assessments.

Table 2 presents various erythrocyte-based assays that allow for the specific evaluation of cellular damage caused by bioactive compounds or materials in contact with blood. Induced, thermal, and cold hemolysis tests help detect alterations in the erythrocyte membrane under different conditions. A value equal to or below 5% is associated with compatibility, whereas higher percentages indicate progressive damage, ranging from moderate to severe. Induced hemolysis is a crucial parameter in determining the biological activity of natural extracts. Due to the lack of clear criteria in the literature to determine whether a natural extract exhibits hemolytic activity, we propose the classification outlined in Table 3.

Table 1. Hemocompatibility test parameters.

Group	Test	°C	λ (nm)	Incubation Time	Centrifugation	Volume (μL)	Positive Control	Negative Control	Reference
Hemolysis	Induced hemolysis	37	540	1–2 h	3000 rpm, 5 min	200–500	Distilled water	Isotonic saline solution	[54,55,62,63]
	Heat-induced hemolysis	50–60	540	30 min	3000 rpm, 5 min	200–500	Distilled water at 60 °C	PBS at 37 °C	[55]
	Cold-induced hemolysis	0–4	540	30 min	3000 rpm, 5 min	200–500	Thermal shock (quick-freezing)	PBS at 37 °C	[55]
Oxidative	Oxidative damage by H ₂ O ₂	37	540	30–60 min	3000 rpm, 5 min	200–500	H ₂ O ₂ (100–500 μM)	Red blood cells in PBS	[56]
	Hemoprotection by APPH	37	540	60–120 min	3000 rpm, 5 min	200–500	APPH	PBS or known protective compound	[64]
	TBARS	95	532	60 min	3500 rpm, 10 min	200–300	Fe ²⁺ + ascorbic acid	Oxidizer-free PBS	[56]
	Xylenol orange (FOX)	RT of 37	540	30–60 min	3000 rpm, 5 min	200–300	H ₂ O ₂ or oxidized lipids	Peroxide-free solution	[57]
Osmotic	Osmotic damage	37	540	30–60 min	3000 rpm, 5 min	200–500	Hypotonic solution (0.1–0.3% NaCl)	Isotonic saline solution (0.9% NaCl)	[55]
Aggregation	Albumin antiaggregant	37	40X	30–60 min	Mild agitation	200–500	Albumin (high concentration)	Untreated erythrocytes	[58]
Function	Methemoglobin	37	630	30 min	3000 rpm, 5 min	200–500	NaNO ₂ (1 mM)	Red blood cells in PBS	[59]
	Anti-methemoglobin	37	630	30–60 min	3000 rpm, 5 min	200–500	NaNO ₂ + control compound	NaNO ₂	[65]

Table 2. Parameters for the interpretation of classical erythrocyte-based assays for the toxicological evaluation of bioactive compounds and biomaterials.

Test	Damage Type	% Negative Control	% Positive Control	Interpretation Criteria	Suggested Controls	Biological Interpretation/Application
Induced Hemolysis	Hemolysis	≤5%	≥90%	<5%: biocompatible 5–25%: moderate damage >25%: hemolytic	PBS (negative), distilled water (positive)	Evaluates red blood cell membrane integrity. Useful for determining if a material, extract, or compound is toxic to blood cells.

Table 2. Cont.

Test	Damage Type	% Negative Control	% Positive Control	Interpretation Criteria	Suggested Controls	Biological Interpretation/Application
Heat Hemolysis	Hemolysis	≤5%	≥90%	<5%: thermally stable membrane >25%: thermal lability	Erythrocytes at 50 °C in PBS	Identifies if a compound increases or reduces thermal stability of membranes. Relevant for bioproducts stored or applied at 37–60 °C.
Cold Hemolysis	Hemolysis	≤5%	≥90%	<5%: cold resistant 5–25%: intermediate >25%: cryolesion	PBS at 4 °C (negative), rapid freezing (positive)	Detects membrane sensitivity to low temperatures. Useful for cell preservation and cryopreservation applications.
Oxidative Damage by H ₂ O ₂	Oxidative	≤10%	≥80%	<10%: antioxidant 10–40%: moderate >40%: pro-oxidant	PBS (negative), H ₂ O ₂ (positive), Trolox (protective)	Evaluates if the compound protects against oxidative stress. Relevant in degenerative diseases, inflammation, and aging.
Hemoprotection by APPH	Oxidative	≤10%	≥70%	<10%: protective 10–30%: intermediate >30%: no protection	APPH alone (positive), Trolox or antioxidants (negative)	Indicates whether a compound prevents chronic lipid oxidation. Useful in therapeutic antioxidant design.
TBARS (MDA)	Oxidative	<0.1 nmol MDA/mL	>0.5 nmol MDA/mL	<0.1: antioxidant 0.1–0.3: intermediate >0.3: pro-oxidant	Fe ²⁺ + ascorbic acid (positive), PBS (negative)	Measures lipid peroxidation. Serves as a marker of cellular damage. Relevant in toxicology, oxidative stress, and supplement development.
Xylenol Orange (FOX)	Oxidative	≤0.1 abs	≥0.3 abs	<0.1: antioxidant 0.1–0.2: moderate >0.2: oxidant	Peroxide-free solution (negative), H ₂ O ₂ or oxidized lipids (positive)	Detects peroxide accumulation. Useful for validating functional foods, natural antioxidants, or cosmetics.
Osmotic Damage	Osmotic	≤5%	≥90%	<5%: compatible 5–25%: partial damage >25%: cell rupture	0.9% NaCl (negative), 0.1–0.3% NaCl (positive)	Evaluates if a compound alters osmotic balance. Important for intravenous solution development and blood-contacting materials.
Anti-aggregation by Albumin	Aggregation	0% aggregated	≥70% aggregated	<10%: anti-aggregating effect 10–40%: intermediate >40%: pro-aggregating	Erythrocytes + albumin (positive), untreated cells (negative)	Evaluates whether a compound prevents or promotes cell aggregation. Relevant for biomaterials or antithrombotic candidates.
Methemoglobin (MetHb)	Functional	<1%	>20%	<1%: no effect 1–10%: mild >10%: heme dysfunction	NaNO ₂ (positive), PBS (negative)	Detects hemoglobin oxidation. Useful for evaluating side effects of drugs or compounds on oxygen transport.
Anti-methemoglobin	Functional	>80% reversal	<20% reversal	>80%: protective 40–80%: moderate <40%: no effect	NaNO ₂ + antioxidant (protective negative), NaNO ₂ alone (positive)	Measures the ability to reverse hemoglobin damage. Relevant in the search for blood-protective or therapeutic agents.

Table 3. Interpretation of hemolytic IC₅₀ values for natural extracts.

IC ₅₀ (µg/mL)	Interpretation
<10 µg/mL	Highly hemolytic
10–200 µg/mL	Hemolytic
200–500 µg/mL	Moderately hemolytic
500–1000 µg/mL	Slightly hemolytic/Tolerable
>1000 µg/mL	Non-hemolytic/Biocompatible

On the other hand, oxidative assays include peroxide-induced models (H₂O₂, AAPH) and methods that quantify lipid peroxidation products (TBARS, FOX). Compounds that keep damage levels below 10% or 0.1 nmol/mL are considered protective, while intermediate or high values indicate oxidative effects [10,17,35,56]. These tests allow for classification of compounds based on their ability to mitigate or induce oxidative stress at the cellular level.

The osmotic damage test evaluates erythrocyte sensitivity in hypotonic solutions. Integrity is maintained with less than 5% lysis, while higher percentages reflect disruption of cellular homeostasis. In parallel, the albumin-induced aggregation test identifies whether a compound promotes or prevents cellular agglutination, with thresholds that help determine pro- or anti-aggregating effects.

Regarding hemoglobin functionality, assays for methemoglobin formation and reversion determine the degree of oxidation of the heme group. Low values indicate a protective effect, while lack of reversion reflects a compound's inability to counteract the damage. This information is key to distinguishing between adverse effects and potentially therapeutic activities.

In this context, the protective or antihemolytic ability of a compound is assessed by its capacity to reduce hemoglobin release compared with oxidant-treated controls. This effect is typically expressed as a percentage of hemolysis inhibition and allows the calculation of concentration–response relationships, from which IC₅₀ values can be derived. Therefore, the IC₅₀ values reported in Table 3 reflect the concentration required to reduce hemolysis by 50% under the specified oxidative conditions, providing a quantitative measure of erythrocyte protection.

4. Application of Erythrocyte-Based Hemocompatibility Tests

Table 4 summarizes the main types of compounds and materials that have been evaluated using hemocompatibility-related assays. A wide variety of compounds have been evaluated under thermal stress conditions to determine their influence on erythrocyte membrane stability. In these assays, red blood cells are subjected to controlled heat exposure to induce membrane destabilization, and test compounds are assessed for their ability to reduce temperature-induced hemolysis. Thus, the readout reflects whether a compound exacerbates membrane damage or confers a protective effect against thermally induced stress, rather than directly alleviating heat stress itself [10]. For example, extracts of *Ibervillea sonora* and *Scenedesmus* sp. have been evaluated against murine lymphoma cells (L5178Y-R), while silver–chitosan nanoparticles have been analyzed for their antimicrobial activity and hemocompatibility. Liposomal delivery systems with endophytic extracts have also been explored in glioblastoma models. Polymers such as chitosan, PEG (polyethylene glycol), and PLGA (polylactic-co-glycolic acid) are frequently studied in combination with bioactive compounds for topical or biomedical applications. In addition, cosmetic formulations and adjuvants have been assessed through oxidative stress and aggregation assays to predict their irritant or immunostimulatory potential. Finally, materials intended for biomedical devices, such as catheters or sensors, are commonly evaluated through contact hemolysis and thermal compatibility tests. These approaches highlight the importance of early biocompatibility assessments in the development of new therapeutic or diagnostic platforms.

Table 4. Application of erythrocyte-based hemocompatibility tests.

Type of Composite/Material	Common Tests Applied	Examples of Application or Study	Reference
Plant and microbial extracts	Hemolysis, antihemolysis (oxidative/osmotic), Free Oxidative Capacity (FOC)	Extracts of <i>Ibervillea sonorae</i> and <i>Scenedesmus</i> sp. tested against L5178Y-R	[66,67]
Metallic nanoparticles (Ag, Au, ZnO, and Fe ₃ O ₄)	Hemolysis, red cell aggregation, heat/cold tests	Silver–chitosan NPs with antimicrobial action and pharmacological activity of AgNPs-EPI	[68,69]
Liposomes and drug delivery systems	Hemolysis, antihemolysis, induced aggregation	Liposomes with endophytic extracts in glioblastoma models and quercetin liposomes for block pancreatic cancer	[70,71]
Polymers and biopolymers (chitosan, PEG, and PLGA)	Hemolysis, FOC, osmotic tests	Hydrogels with chitosan and plant extracts for topical applications and chitosan nanoparticles for the codelivery of CBD and BDNF for Alzheimer’s Disease	[72,73]
Synthetic or semi-synthetic compounds	Hemolysis, aggregation, lipid peroxidation	Synthetic derivatives with anticancer, antibacterial or antiparasitic potential	[74,75]
Cosmetic or topical formulations	Hemolysis, simulated irritation, oxidative stress	Creams and latex dressing with natural extracts evaluated in in vitro models	[76,77]
Antimicrobial agents	Hemolysis, antihemolysis by H ₂ O ₂ , FOC	Hemocompatible activity of antibacterial compounds of plant origin and antimicrobial electrospun nanofiber dressings	[78,79]
Adjuvants and immunostimulants	Aggregation, oxidative activation, thermal stress	Emulsions with saponins as vaccine adjuvants	[80,81]
Materials for biomedical devices	Contact hemolysis, aggregation, thermal compatibility	In vitro evaluation of polymers for coatings on catheters or sensors	[82,83]

5. Discussion

The search for safe and effective bioactive compounds in areas such as pharmacognosy, functional foods, and nanomaterials requires efficient experimental models that highlight accessibility, sensitivity, and physiological relevance to estimate the biocompatibility of nanomaterials, as well as bioactive compounds, natural or synthetic [84,85]. From this context, a stable, reliable, and representative scaffold of the behavior of cell membranes when exposed to physical or chemical agents is essential [10]. Based on this, classical assays with red blood cells provide critical information on membrane stability and allow the evaluation of the potential hematic cytotoxicity of natural extracts, secondary metabolites, or nanostructured systems [16]. These tests, by quantifying the release of hemoglobin or evaluating the protection against oxidative, osmotic or thermal damage, allow an early classification of the potential risk of the compounds. Therefore, their usefulness is consolidated as an initial screening tool in the rational design of bioactive products [9].

In pharmacognosy, extracts derived from plants, fungi, and endophytic microorganisms present diverse chemical structures, whose interactions with the cell membrane are difficult to predict during the early stages and subsequent stages of the analysis of crude

extracts, partitions, and fractions of the bioactive extracts [86]. Assays such as the H₂O₂ antihemolytic test or osmotic fragility are especially useful for identifying compounds with antioxidant or membrane-stabilizing properties, which is desirable not only to reduce toxicity, but also as an indication of possible therapeutic benefits by directing studies from early stages and directing subsequent studies of bioactive extracts and compounds [17].

For functional and nutraceutical ingredients, red blood cells also allow for safety assessment in complex matrices prior to the use of animal models. Detection of subhemolytic or aggregation effects alert us to undesirable interactions that compromise the biosafety of these products. Thus, these tests align with the 3R principles (reduce, refine, and replace) by reducing the use of animal models in preliminary phases [15].

In the field of nanomaterials, such as liposomes, metallic nanoparticles or biofunctional polymers, red blood cells estimate the hematic compatibility of the transport systems [12]. Excessive hemolysis may indicate an unstable surface or an inadequate surface charge [87]. Moreover, the capacity of certain nanostructured compounds to protect or alter the erythrocyte membrane can be correlated with their behavior in blood and predict adverse events such as thrombogenicity or systemic cytotoxicity [88].

It is important to emphasize that, although erythrocytes represent a relatively simple experimental model, their high sensitivity allows for the detection of early adverse effects that may not be evident in more resistant or complex cell systems. This makes them an effective preliminary screening tool prior to scaling up studies to more advanced in vitro models, such as tumor cell lines, or to in vivo animal models [88]. Moreover, clearly defined interpretation criteria for each erythrocyte-based assay, together with rigorously established positive and negative controls, enable a systematic and reliable comparison of the hemolytic profiles of different compounds or materials. Consequently, these methodologies facilitate the early assessment of toxicity and blood compatibility and are particularly valuable during the initial stages of the development of new therapeutic agents or biomaterials, as well as in screening assays [80].

Given these advantages, future studies should aim to further standardize erythrocyte-based assays and expand their use as early screening tools for hemocompatibility and toxicity. Improving methodological consistency, incorporating quantitative endpoints, and validating results across different types of bioactive compounds and nanomaterials will strengthen their predictive value. Emerging analytical approaches, including automated image-based analysis and data-driven tools, may enable the detection of subtle erythrocyte morphological changes beyond conventional hemoglobin release measurements. In pharmacognosy and nutraceutical research, integrating erythrocyte assays with metabolomic profiling could further clarify how specific bioactive fractions interact with and stabilize membrane structures at the molecular level. Additionally, combining red blood cell assays with complementary in vitro models may provide a more comprehensive evaluation of blood compatibility. Together, these advances are expected to enhance the reliability and translational relevance of erythrocyte-based testing and support its broader application in the development of safe therapeutic, nutritional, and nanotechnology-related products.

In conclusion, the use of red blood cells as a biocompatibility model represents an efficient, cost-effective, and reproducible strategy for the preliminary toxicological evaluation of bioactive compounds. Its broad applicability in pharmacognosy, functional ingredient design, and nanomaterial development positions erythrocyte-based assays as a critical step toward the development of safe products with therapeutic or nutritional potential. Beyond their current use, the re-evaluation and further standardization of these assays, together with the incorporation of quantitative endpoints, automation, and complementary in vitro and in silico approaches, may substantially enhance their predictive capacity. Although erythrocyte-based assays have been widely applied in hemocompatibility and toxicity test-

ing, their integration into predictive or standardized screening frameworks remains limited. This is not due to a lack of biological relevance, but rather to methodological heterogeneity, variability in erythrocyte preparation and assay conditions, and the absence of harmonized acceptance criteria across laboratories. In addition, erythrocytes are often regarded as oversimplified models, which has restricted their broader adoption despite their sensitivity to early membrane and oxidative damage. Addressing these limitations may help optimize early-stage screening strategies, reduce reliance on animal models, and support translational research across areas such as healthcare, food science, and nanotechnology.

Within a comprehensive biocompatibility and toxicity assessment framework, erythrocyte-based assays are best positioned as early-stage screening tools. Their simplicity and sensitivity to membrane and oxidative damage enable rapid identification of potentially harmful compounds. Findings from these assays can subsequently be complemented by more complex in vitro systems, including nucleated blood cells, endothelial models, and immune-based assays, and, when necessary, by ex vivo or in vivo studies. In this tiered strategy, erythrocyte assays do not replace advanced models but support informed decision-making by prioritizing candidates and reducing unnecessary downstream testing.

6. Conclusions

Red blood cell-based hemocompatibility assays provide a sensitive model for the early evaluation of interactions between bioactive compounds, biomaterials, and cell membranes. As discussed in this review, erythrocytes allow the assessment of diverse damage mechanisms, including membrane disruption, oxidative stress, and regulated clearance processes. When key experimental variables are properly controlled, these assays offer meaningful mechanistic insight and predictive value, supporting their use as an initial screening tool in pharmacological and toxicological studies.

Author Contributions: Conceptualization, C.I.R.-S. and N.E.R.-G.; investigation, A.L.D.-M., D.L.C.-P., A.G., B.E.C.-V. and A.R.C.-G.; writing—original draft preparation, C.I.R.-S., N.E.R.-G. and R.G.-F.; writing—review and editing, P.T.-G., C.I.R.-S. and R.G.-F.; supervision, C.M.Q.-F. and R.G.-F.; funding acquisition, C.I.R.-S., N.E.R.-G. and R.G.-F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Consejo Nacional de Humanidades, Ciencia y Tecnología 445572 to C.I.R.-S.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Bashir, S.; Fitaihi, R.; Abdelhakim, H.E. Advances in Formulation and Manufacturing Strategies for the Delivery of Therapeutic Proteins and Peptides in Orally Disintegrating Dosage Forms. *Eur. J. Pharm. Sci.* **2023**, *182*, 106374. [[CrossRef](#)] [[PubMed](#)]
2. Joseph, T.M.; Kar Mahapatra, D.; Esmaeili, A.; Piszczczyk, Ł.; Hasanin, M.S.; Kattali, M.; Haponiuk, J.; Thomas, S. Nanoparticles: Taking a Unique Position in Medicine. *Nanomaterials* **2023**, *13*, 574. [[CrossRef](#)] [[PubMed](#)]
3. Uddin, T.M.; Chakraborty, A.J.; Khusro, A.; Zidan, B.R.M.; Mitra, S.; Bin Emran, T.; Dhama, K.; Ripon, M.K.H.; Gajdács, M.; Sahibzada, M.U.K.; et al. Antibiotic Resistance in Microbes: History, Mechanisms, Therapeutic Strategies and Future Prospects. *J. Infect. Public Health* **2021**, *14*, 1750–1766. [[CrossRef](#)] [[PubMed](#)]
4. Gupta, R.; Sharma, S. Role of Alternatives to Antibiotics in Mitigating the Antimicrobial Resistance Crisis. *Indian J. Med. Res.* **2022**, *156*, 464–477. [[CrossRef](#)]
5. Alghareeb, S.A.; Alfhili, M.A.; Fatima, S. Molecular Mechanisms and Pathophysiological Significance of Eryptosis. *Int. J. Mol. Sci.* **2023**, *24*, 5079. [[CrossRef](#)]

6. Pérez-Pacheco, A.; Salazar-Nieva, L.B.; García-Valenzuela, A.; Mendoza-García, E.R.; Martínez-Tovar, A.; Olarte-Carrillo, I.; Márquez-Islas, R. Erythrocytes' Osmotic Fragility Test with a Standard Abbe Refractometer. *Rev. Médica Hosp. Gen. México* **2020**, *83*, 20–25. [\[CrossRef\]](#)
7. Griesinger, C.; Desprez, B.; Coecke, S.; Casey, W.; Zuang, V. Validation of Alternative In Vitro Methods to Animal Testing: Concepts, Challenges, Processes and Tools. *Adv. Exp. Med. Biol.* **2016**, *856*, 65–132. [\[CrossRef\]](#)
8. Cheng, C.-M.; Kuan, C.-M.; Chen, C.-F. Low-Cost In Vitro Diagnostic Technologies. In *In-Vitro Diagnostic Devices: Introduction to Current Point-of-Care Diagnostic Devices*; Springer International Publishing: Cham, Switzerland, 2016; pp. 59–91. [\[CrossRef\]](#)
9. Farag, M.R.; Alagawany, M. Erythrocytes as a Biological Model for Screening of Xenobiotics Toxicity. *Chem. Biol. Interact.* **2018**, *279*, 73–83. [\[CrossRef\]](#)
10. Podsiedlik, M.; Markowicz-Piasecka, M.; Sikora, J. Erythrocytes as Model Cells for Biocompatibility Assessment, Cytotoxicity Screening of Xenobiotics and Drug Delivery. *Chem. Biol. Interact.* **2020**, *332*, 109305. [\[CrossRef\]](#)
11. Weber, M.; Steinle, H.; Golombek, S.; Hann, L.; Schlensak, C.; Wendel, H.P.; Avci-Adali, M. Blood-Contacting Biomaterials: In Vitro Evaluation of the Hemocompatibility. *Front. Bioeng. Biotechnol.* **2018**, *6*, 99. [\[CrossRef\]](#)
12. De La Harpe, K.M.; Kondiah, P.P.D.; Choonara, Y.E.; Marimuthu, T.; Du Toit, L.C.; Pillay, V. The Hemocompatibility of Nanoparticles: A Review of Cell–Nanoparticle Interactions and Hemostasis. *Cells* **2019**, *8*, 1209. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Malehmir, S.; Esmaili, M.A.; Khaksary Mahabady, M.; Sobhani-Nasab, A.; Atapour, A.; Ganjali, M.R.; Ghasemi, A.; Moradi Hasan-Abad, A. A Review: Hemocompatibility of Magnetic Nanoparticles and Their Regenerative Medicine, Cancer Therapy, Drug Delivery, and Bioimaging Applications. *Front. Chem.* **2023**, *11*, 1249134. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Blok, S.L.J.; Engels, G.E.; van Oeveren, W. In Vitro Hemocompatibility Testing: The Importance of Fresh Blood. *Biointerphases* **2016**, *11*, 029802. [\[CrossRef\]](#) [\[PubMed\]](#)
15. von Petersdorff-Campen, K.; Daners, M.S. Hemolysis Testing In Vitro: A Review of Challenges and Potential Improvements. *ASAIO J.* **2022**, *68*, 3–13. [\[CrossRef\]](#)
16. Sæbø, I.P.; Bjørås, M.; Franzyk, H.; Helgesen, E.; Booth, J.A. Optimization of the Hemolysis Assay for the Assessment of Cytotoxicity. *Int. J. Mol. Sci.* **2023**, *24*, 2914. [\[CrossRef\]](#)
17. Shevchenko, O.G. Blood Erythrocytes—A Biological Model for Evaluating Antioxidant Activity of Chemical Compounds (A Review). *Russ. J. Bioorganic Chem.* **2024**, *50*, 2191–2208. [\[CrossRef\]](#)
18. Wang, X.; Wang, X. Overview on Biocompatibilities of Implantable Biomaterials. In *Advances in Biomaterials Science and Biomedical Applications*; IntechOpen: London, UK, 2013. [\[CrossRef\]](#)
19. Turpin, C.; Catan, A.; Meilhac, O.; Bourdon, E.; Canonne-Hergaux, F.; Rondeau, P. Erythrocytes: Central Actors in Multiple Scenes of Atherosclerosis. *Int. J. Mol. Sci.* **2021**, *22*, 5843. [\[CrossRef\]](#)
20. Li, N.; Chen, S.; Xu, K.; He, M.T.; Dong, M.Q.; Zhang, Q.C.; Gao, N. Structural Basis of Membrane Skeleton Organization in Red Blood Cells. *Cell* **2023**, *186*, 1912–1929.e18. [\[CrossRef\]](#)
21. Nigra, A.D.; Casale, C.H.; Santander, V.S. Human Erythrocytes: Cytoskeleton and Its Origin. *Cell. Mol. Life Sci.* **2020**, *77*, 1681–1694. [\[CrossRef\]](#)
22. Jelokhani-Niaraki, M. Membrane Proteins: Structure, Function and Motion. *Int. J. Mol. Sci.* **2023**, *24*, 468. [\[CrossRef\]](#)
23. Sae-Lee, W.; McCafferty, C.L.; Verbeke, E.J.; Havugimana, P.C.; Papoulas, O.; McWhite, C.D.; Houser, J.R.; Vanuytsel, K.; Murphy, G.J.; Drew, K.; et al. The Protein Organization of a Red Blood Cell. *Cell Rep.* **2022**, *40*, 111103. [\[CrossRef\]](#)
24. Duchnowicz, P.; Pilarski, R.; Michałowicz, J.; Bukowska, B. Changes in Human Erythrocyte Membrane Exposed to Aqueous and Ethanolic Extracts from *Uncaria Tomentosa*. *Molecules* **2021**, *26*, 3189. [\[CrossRef\]](#)
25. Maruyama, T.; Hieda, M.; Mawatari, S.; Fujino, T. Rheological Abnormalities in Human Erythrocytes Subjected to Oxidative Inflammation. *Front. Physiol.* **2022**, *13*, 837926. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Ruiz, J.; Calderon, J.; Rondón-Villarreal, P.; Torres, R. Analysis of Structure and Hemolytic Activity Relationships of Antimicrobial Peptides (AMPs). In *Proceedings of the Advances in Computational Biology: Proceedings of the 2nd Colombian Congress on Computational Biology and Bioinformatics (CCBCOL), Manizales, Colombia, 25–27 September 2013*; Springer International Publishing: Cham, Switzerland, 2014; Volume 232, pp. 253–258. [\[CrossRef\]](#)
27. Li, J.; Koh, J.J.; Liu, S.; Lakshminarayanan, R.; Verma, C.S.; Beuerman, R.W. Membrane Active Antimicrobial Peptides: Translating Mechanistic Insights to Design. *Front. Neurosci.* **2017**, *11*, 73. [\[CrossRef\]](#)
28. Obeagu, E.I.; Igwe, M.C.; Obeagu, G.U. Oxidative Stress's Impact on Red Blood Cells: Unveiling Implications for Health and Disease. *Medicine* **2024**, *103*, E37360. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Polyakov, N.; Leshina, T.; Fedenok, L.; Slepneva, I.; Kirilyuk, I.; Furso, J.; Olchawa, M.; Sarna, T.; Elas, M.; Bilkis, I.; et al. Redox-Active Quinone Chelators: Properties, Mechanisms of Action, Cell Delivery, and Cell Toxicity. *Antioxid. Redox Signal.* **2018**, *28*, 1394–1403. [\[CrossRef\]](#)
30. Petty, H.R.; Zhou, M.-J.; Zheng, Z. Oxidative Damage by Phenylhydrazine Diminishes Erythrocyte Anion Transport. *Biochim. Biophys. Acta (BBA)—Biomembr.* **1991**, *1064*, 308–314. [\[CrossRef\]](#)

31. Ansari, F.A.; Ali, S.N.; Mahmood, R. Sodium Nitrite-Induced Oxidative Stress Causes Membrane Damage, Protein Oxidation, Lipid Peroxidation and Alters Major Metabolic Pathways in Human Erythrocytes. *Toxicol. In Vitro* **2015**, *29*, 1878–1886. [\[CrossRef\]](#)
32. Spinelli, S.; Marino, A.; Remigante, A.; Morabito, R. Redox Homeostasis in Red Blood Cells: From Molecular Mechanisms to Antioxidant Strategies. *Curr. Issues Mol. Biol.* **2025**, *47*, 655. [\[CrossRef\]](#)
33. Kriebardis, A.G.; Antonelou, M.H.; Stamoulis, K.E.; Economou-Petersen, E.; Margaritis, L.H.; Papassideri, I.S. Progressive Oxidation of Cytoskeletal Proteins and Accumulation of Denatured Hemoglobin in Stored Red Cells. *J. Cell. Mol. Med.* **2007**, *11*, 148–155. [\[CrossRef\]](#)
34. Carmo de Carvalho e Martins, M.; Martins; da Silva Santos Oliveira, A.S.; da Silva, L.A.A.; Primo, M.G.S.; de Carvalho Lira, V.B. Biological Indicators of Oxidative Stress [Malondialdehyde, Catalase, Glutathione Peroxidase, and Superoxide Dismutase] and Their Application in Nutrition. In *Biomarkers in Nutrition*; Springer International Publishing: Cham, Switzerland, 2022; pp. 1–25. [\[CrossRef\]](#)
35. Fujii, J.; Homma, T.; Kobayashi, S.; Warang, P.; Madkaikar, M.; Mukherjee, M.B. Erythrocytes as a Preferential Target of Oxidative Stress in Blood. *Free Radic. Res.* **2021**, *55*, 781–799. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Mariano, A.; Bigioni, I.; Misiti, F.; Fattorini, L.; d’Abusco, A.S.; Rodio, A. The Nutraceuticals as Modern Key to Achieve Erythrocyte Oxidative Stress Fighting in Osteoarthritis. *Curr. Issues Mol. Biol.* **2022**, *44*, 3481–3495. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Morera, D.; MacKenzie, S.A. Is There a Direct Role for Erythrocytes in the Immune Response? *Vet. Res.* **2011**, *42*, 89. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Anderson, H.L.; Brodsky, I.E.; Mangalmurti, N.S. The Evolving Erythrocyte: RBCs as Modulators of Innate Immunity. *J. Immunol.* **2018**, *201*, 1343. [\[CrossRef\]](#)
39. van Kasteren, S.I.; Neeffes, J.; Ova, H. Creating Molecules That Modulate Immune Responses. *Nat. Rev. Chem.* **2018**, *2*, 184–193. [\[CrossRef\]](#)
40. Aljabali, A.A.; Obeid, M.A.; Bashatwah, R.M.; Serrano-Aroca, Á.; Mishra, V.; Mishra, Y.; El-Tanani, M.; Hromić-Jahjefendić, A.; Kapoor, D.N.; Goyal, R.; et al. Nanomaterials and Their Impact on the Immune System. *Int. J. Mol. Sci.* **2023**, *24*, 2008. [\[CrossRef\]](#)
41. Anliker, M.; Schmidt, C.Q.; Harder, M.J.; Ganchev, G.; von Zabern, I.; Höchsmann, B.; Schrezenmeier, H.; Weinstock, C. Complement Activation by Human Red Blood Cell Antibodies: Hemolytic Potential of Antibodies and Efficacy of Complement Inhibitors Assessed by a Sensitive Flow Cytometric Assay. *Transfusion* **2018**, *58*, 2992–3002. [\[CrossRef\]](#)
42. Hair, P.; Goldman, D.W.; Li, J.; Petri, M.; Krishna, N.; Cunnion, K. Classical Complement Activation on Human Erythrocytes in Subjects with Systemic Lupus Erythematosus and a History of Autoimmune Hemolytic Anemia. *Lupus* **2020**, *29*, 1179–1188. [\[CrossRef\]](#)
43. Tkachenko, A.; Alfihili, M.A.; Alsughayyir, J.; Attanzio, A.; Al Mamun Bhuyan, A.; Bukowska, B.; Cilla, A.; Quintanar-Escorza, M.A.; Föller, M.; Havranek, O.; et al. Current Understanding of Eryptosis: Mechanisms, Physiological Functions, Role in Disease, Pharmacological Applications, and Nomenclature Recommendations. *Cell Death Dis.* **2025**, *16*, 467. [\[CrossRef\]](#)
44. Agrawal, R.; Sherwood, J.; Chhablani, J.; Ricchiarri, A.; Kim, S.; Jones, P.H.; Balabani, S.; Shima, D. Red Blood Cells in Retinal Vascular Disorders. *Blood Cells Mol. Dis.* **2016**, *56*, 53–61. [\[CrossRef\]](#)
45. Abbasi, M.; Farutin, A.; Ez-Zahraouy, H.; Benyoussef, A.; Misbah, C. Erythrocyte-Erythrocyte Aggregation Dynamics under Shear Flow. *Phys. Rev. Fluids* **2021**, *6*, 023602. [\[CrossRef\]](#)
46. Hasirci, V.; Hasirci, N. Hemocompatibility. In *Fundamentals of Biomaterials*; Springer: Berlin/Heidelberg, Germany, 2024; pp. 205–217. [\[CrossRef\]](#)
47. Manivasagam, V.K.; Sabino, R.M.; Kantam, P.; Popat, K.C. Surface Modification Strategies to Improve Titanium Hemocompatibility: A Comprehensive Review. *Mater. Adv.* **2021**, *2*, 5824–5842. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Seyfert, U.T.; Biehl, V.; Schenk, J. In Vitro Hemocompatibility Testing of Biomaterials According to the ISO 10993-4. *Biomol. Eng.* **2002**, *19*, 91–96. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Harisa, G.I.; Badran, M.M.; AlQahtani, S.A.; Alanazi, F.K.; Attia, S.M. Pravastatin Chitosan Nanogels-Loaded Erythrocytes as a New Delivery Strategy for Targeting Liver Cancer. *Saudi Pharm. J.* **2016**, *24*, 74–81. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Elblbesy, M.A.; Elblbesy, M.A. Hemocompatibility of Albumin Nanoparticles as a Drug Delivery System—An in Vitro Study. *J. Biomater. Nanobiotechnol.* **2016**, *7*, 64–71. [\[CrossRef\]](#)
51. Henkelman, S.; Rakhorst, G.; Blanton, J.; van Oeveren, W. Standardization of Incubation Conditions for Hemolysis Testing of Biomaterials. *Mater. Sci. Eng. C* **2009**, *29*, 1650–1654. [\[CrossRef\]](#)
52. Dobrovol'skaia, M.A.; McNeil, S.E. Immunological Properties of Engineered Nanomaterials. *Nat. Nanotechnol.* **2007**, *2*, 469–478. [\[CrossRef\]](#)
53. Tkachenko, A. Hemocompatibility Studies in Nanotoxicology: Hemolysis or Eryptosis? (A Review). *Toxicol. In Vitro* **2024**, *98*, 105814. [\[CrossRef\]](#)
54. Delgado-Miranda, A.L.; Gomez-Flores, R.; Rodríguez-Garza, N.E.; Pérez-González, O.; Tamez-Guerra, P.; Caballero-Hernández, D.; Clark-Pérez, D.L.; Quintanilla-Licea, R.; García, A.; Romo-Sáenz, C.I. In Vitro Anti-Glioblastoma Activity of *Echinocereus engelmannii*- and *Echinocereus pectinatus*-Associated Bacterial Endophyte Extracts. *Life* **2025**, *15*, 519. [\[CrossRef\]](#)

55. Mendez-Encinas, M.A.; Valencia, D.; Ortega-García, J.; Carvajal-Millan, E.; Díaz-Ríos, J.C.; Mendez-Pfeiffer, P.; Soto-Bracamontes, C.M.; Garibay-Escobar, A.; Alday, E.; Velazquez, C. Anti-Inflammatory Potential of Seasonal Sonoran Propolis Extracts and Some of Their Main Constituents. *Molecules* **2023**, *28*, 4496. [\[CrossRef\]](#)
56. Benincasa, G.; Bontempo, P.; Trama, U.; Napoli, C. Synergistic Effects of a Novel Combination of Natural Compounds Prevent H₂O₂-Induced Oxidative Stress in Red Blood Cells. *Int. J. Mol. Sci.* **2025**, *26*, 1334. [\[CrossRef\]](#)
57. Pinto, M.D.C.; Tejeda, A.; Duque, A.L.; Macías, P. Determination of Lipoygenase Activity in Plant Extracts Using a Modified Ferrous Oxidation–Xylenol Orange Assay. *J. Agric. Food Chem.* **2007**, *55*, 5956–5959. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Ben-Ami, R.; Barshtein, G.; Mardi, T.; Deutch, V.; Elkayam, O.; Yedgar, S.; Berliner, S. A Synergistic Effect of Albumin and Fibrinogen on Immunoglobulin-Induced Red Blood Cell Aggregation. *Am. J. Physiol.-Heart Circ. Physiol.* **2003**, *285*, H2663–H2669. [\[CrossRef\]](#) [\[PubMed\]](#)
59. Chiodi, H.; Mohler, J.G. With the Technical Assistance of Manuel, D.S. Nonautocatalytic Methemoglobin Formation by Sodium Nitrite under Aerobic and Anaerobic Conditions. *Environ. Res.* **1987**, *44*, 45–55. [\[CrossRef\]](#) [\[PubMed\]](#)
60. Chikezie, P.C. Methaemoglobin Content and NADH-Methaemoglobin Reductase Activity of Three Human Erythrocyte Genotypes. *Asian J. Biochem.* **2011**, *6*, 98–103. [\[CrossRef\]](#)
61. Ansari, F.A.; Ali, S.N.; Mahmood, R. Crocin Protects Human Erythrocytes from Nitrite-Induced Methemoglobin Formation and Oxidative Damage. *Cell Biol. Int.* **2016**, *40*, 1320–1331. [\[CrossRef\]](#)
62. Domínguez-Gámez, M.; Romo-Sáenz, C.I.; Gomez-Flores, R.; González-Ochoa, G.; García-Romero, A.; Orozco-Flores, A.A.; Rodríguez-Padilla, C.; Tamez-Guerra, P. In Vitro Antitumor, Antioxidant, and Hemolytic Activities of *Chlorella Sorokiniana* Methanol Extracts and Collective Fractions. *Appl. Sci.* **2024**, *14*, 9613. [\[CrossRef\]](#)
63. Rodríguez-Garza, N.E.; Quintanilla-Licea, R.; Romo-Sáenz, C.I.; Elizondo-Luevano, J.H.; Tamez-Guerra, P.; Rodríguez-Padilla, C.; Gomez-Flores, R. In Vitro Biological Activity and Lymphoma Cell Growth Inhibition by Selected Mexican Medicinal Plants. *Life* **2023**, *13*, 958. [\[CrossRef\]](#)
64. Clark-Pérez, D.L.; Romo-Sáenz, C.I.; Ramírez-Villalobos, J.M.; Tamez-Guerra, P.; Caballero-Hernández, D.; Delgado-Miranda, A.L.; García, A.; Elizondo-Luevano, J.H.; Rodríguez-Padilla, C.; Gomez-Flores, R. In Vitro and In Vivo Antitumor Activity of *Lophocereus marginatus* (DC.) S. Arias & Terrazas Endophytic *Aspergillus versicolor* and *Metarhizium anisopliae* Extracts Against the Murine Lymphoma L5178Y-R. *Microorganisms* **2024**, *12*, 2310. [\[CrossRef\]](#)
65. Das, D.; Sen, S.; Sen, K. Caffeine and Catechin towards Prevention of Drug Induced Oxidation of Hemoglobin: A Spectroscopic Study. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2020**, *232*, 118167. [\[CrossRef\]](#)
66. Romero-Arguelles, R.; Romo-Sáenz, C.I.; Tamez-Guerra, P.; Fonseca-Rivera, D.; Elizondo-Luevano, J.H.; Rodríguez-Garza, N.E.; Pérez-González, O.; Quiñones-Flores, C.M.; Arzate-Quintana, C.; Hernandez, L.P.L.; et al. In Vitro and In Vivo Antitumor Activity of a Chloroform Partition from *Ibervillea sonora* (S. Watson) Greene Endophytic *Bacillus subtilis* Extracts. *Plants* **2025**, *14*, 1474. [\[CrossRef\]](#) [\[PubMed\]](#)
67. Fonseca-Rivera, D.; Caballero-Hernández, D.E.; Tamez-Guerra, P.; Romero-Arguelles, R.; Elizondo-Luevano, J.H.; Clark-Perez, D.L.; Quiñones-Flores, C.M.; Castillo-Gonzalez, A.R.; Gomez-Flores, R.; Romo-Sáenz, C.I. Nutraceutical and Antitumoral Potential of *Scenedesmus* Sp. in In Vitro and In Vivo Models. *Foods* **2026**, *15*, 186. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Luna-Vázquez-gómez, R.; Arellano-García, M.E.; Toledano-Magaña, Y.; García-Ramos, J.C.; Radilla-Chávez, P.; Salas-Vargas, D.S.; Casillas-Figueroa, F.; Ruiz-Ruiz, B.; Pestryakov, A.; Bogdanchikova, N. Bell Shape Curves of Hemolysis Induced by Silver Nanoparticles: Review and Experimental Assay. *Nanomaterials* **2022**, *12*, 1066. [\[CrossRef\]](#) [\[PubMed\]](#)
69. Hajtuch, J.; Iwicka, E.; Szczoczarz, A.; Flis, D.; Megiel, E.; Ciecior, P.; Radomski, M.W.; Santos-Martinez, M.J.; Inkielewicz-Stepniak, I. The Pharmacological Effects of Silver Nanoparticles Functionalized with Eptifibatide on Platelets and Endothelial Cells. *Int. J. Nanomed.* **2022**, *17*, 4383. [\[CrossRef\]](#)
70. Ghani, U.; Ghor, F.K.; Qamar, M.U.; Khan, H.; Azad, B.; Habib, S.; Justin, S.; Khan, I.N.; Shah, T.A.; Shazly, G.A.; et al. SLNP-Based CDK4- Targeted Nanotherapy against Glioblastoma. *Front. Oncol.* **2024**, *14*, 1455816. [\[CrossRef\]](#)
71. Sun, G.; Wu, Y.; Li, J.; Yang, M.; Xu, H.; Li, Y.; Tong, P.; Shao, R.; Liu, Y.; Kong, X. Quercetin Liposomes Conjugated with Hyaluronidase: An Efficient Drug Delivery System to Block Pancreatic Cancer. *J. Control. Release* **2025**, *382*, 113642. [\[CrossRef\]](#)
72. Catanzano, O.; Gomez d'Ayala, G.; D'Agostino, A.; Di Lorenzo, F.; Schiraldi, C.; Malinconico, M.; Lanzetta, R.; Bonina, F.; Laurienzo, P. PEG-Crosslinked-Chitosan Hydrogel Films for in Situ Delivery of *Opuntia Ficus-Indica* Extract. *Carbohydr. Polym.* **2021**, *264*, 117987. [\[CrossRef\]](#)
73. Mahanta, A.K.; Chaulagain, B.; Trivedi, R.; Singh, J. Mannose-Functionalized Chitosan-Coated PLGA Nanoparticles for Brain-Targeted Codelivery of CBD and BDNF for the Treatment of Alzheimer's Disease. *ACS Chem. Neurosci.* **2024**, *15*, 4021–4032. [\[CrossRef\]](#)
74. Bhardwaj, N.; Tripathi, N.; Goel, B.; Jain, S.K. Anticancer Activity of Diosgenin and Its Semi-Synthetic Derivatives: Role in Autophagy Mediated Cell Death and Induction of Apoptosis. *Mini-Rev. Med. Chem.* **2021**, *21*, 1646–1665. [\[CrossRef\]](#)

75. Da Rosa, R.; Dambrós, B.P.; Höehr de Moraes, M.; Grand, L.; Jacolot, M.; Popowycz, F.; Steindel, M.; Schenkel, E.P.; Campos Bernardes, L.S. Natural-Product-Inspired Design and Synthesis of Two Series of Compounds Active against *Trypanosoma cruzi*: Insights into Structure–Activity Relationship, Toxicity, and Mechanism of Action. *Bioorganic Chem.* **2022**, *119*, 105492. [[CrossRef](#)]
76. Lee, H.H.; Huang, Y.H.; Huang, J.J.; Huang, M.Y. Exploring Black Soybean Extract Cream for Inflammatory Dermatitis—Toward Radiation Dermatitis Relief. *Int. J. Mol. Sci.* **2024**, *25*, 11598. [[CrossRef](#)] [[PubMed](#)]
77. Herculano, R.D.; dos Santos, T.O.; de Barros, N.R.; Pegorin Brasil, G.S.A.; Scontri, M.; Carvalho, B.G.; Mecwan, M.; Farhadi, N.; Kawakita, S.; Perego, C.H.; et al. Aloe Vera-Loaded Natural Rubber Latex Dressing as a Potential Complementary Treatment for Psoriasis. *Int. J. Biol. Macromol.* **2023**, *242*, 124779. [[CrossRef](#)] [[PubMed](#)]
78. De Lima-Saraiva, S.R.G.; Oliveira, F.G.d.S.; Junior, R.G.d.O.; Araújo, C.d.S.; de Oliveira, A.P.; Pacheco, A.G.M.; Rolim, L.A.; de Amorim, E.L.C.; César, F.C.S.; Almeida, J.R.G.d.S. Chemical Analysis and Evaluation of Antioxidant, Antimicrobial, and Photoprotective Activities of *Schinopsis brasiliensis* Engl. (Anacardiaceae). *Sci. World J.* **2017**, *2017*, 1713921. [[CrossRef](#)] [[PubMed](#)]
79. Nobre, D.; Ekoh, E.; Silva, D.C.; Branco, A.C.; Pais, V.; Almeida, A.; Barroso, H.; Salema-Oom, M.; Figueiro, R.; Colaço, R.; et al. Antimicrobial Casein/Poly(Vinyl Alcohol) Electrospun Nanofibers-Based Dressings. *Int. J. Pharm.* **2025**, *686*, 126320. [[CrossRef](#)]
80. Sharma, R.; Palanisamy, A.; Dhama, K.; Mal, G.; Singh, B.; Singh, K.P. Exploring the Possible Use of Saponin Adjuvants in COVID-19 Vaccine. *Hum. Vaccines Immunother.* **2020**, *16*, 2944–2953. [[CrossRef](#)]
81. Han, Z.; Jin, J.; Chen, X.; He, Y.; Sun, H. Adjuvant Activity of Tubeimosides by Mediating the Local Immune Microenvironment. *Front. Immunol.* **2023**, *14*, 1108244. [[CrossRef](#)]
82. Haktaniyan, M.; Bradley, M. Polymers Showing Intrinsic Antimicrobial Activity. *Chem. Soc. Rev.* **2022**, *51*, 8584–8611. [[CrossRef](#)]
83. Yao, M.; Wei, Z.; Li, J.; Guo, Z.; Yan, Z.; Sun, X.; Yu, Q.; Wu, X.; Yu, C.; Yao, F.; et al. Microgel Reinforced Zwitterionic Hydrogel Coating for Blood-Contacting Biomedical Devices. *Nat. Commun.* **2022**, *13*, 5339. [[CrossRef](#)]
84. Li, X.; Wang, L.; Fan, Y.; Feng, Q.; Cui, F.Z. Biocompatibility and Toxicity of Nanoparticles and Nanotubes. *J. Nanomater.* **2012**, *2012*, 548389. [[CrossRef](#)]
85. Soares, S.; Sousa, J.; Pais, A.; Vitorino, C. Nanomedicine: Principles, Properties, and Regulatory Issues. *Front. Chem.* **2018**, *6*, 356901. [[CrossRef](#)]
86. Rodríguez-Garza, N.E.; Quintanilla-Licea, R.; Gomez-Flores, R.; Galaviz-Silva, L.; Molina-Garza, Z.J. In Vitro Antiprotozoal Activity of *Schinus molle* Extract, Partitions, and Fractions against *Trypanosoma cruzi*. *Plants* **2024**, *13*, 2177. [[CrossRef](#)]
87. Cho, W.S.; Thielbeer, F.; Duffin, R.; Johansson, E.M.V.; Megson, I.L.; Macnee, W.; Bradley, M.; Donaldson, K. Surface Functionalization Affects the Zeta Potential, Coronal Stability and Membranolytic Activity of Polymeric Nanoparticles. *Nanotoxicology* **2014**, *8*, 202–211. [[CrossRef](#)]
88. Li, S.; Yan, S.M.; Zhang, L.W.; Yang, X.Y.; Guo, Z. Blood Compatibility Evaluation of Polydopamine Nanoparticles. *Front. Pharmacol.* **2025**, *16*, 1530650. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.