Article published on 2025-9-10



# Beyond Hemoglobin: A Review of Hemocyanin and the Biology of Purple Blood

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Chinese Journal of Applied Physiology, 2025: e20250023

**Abstract** Hemocyanin is dissolved freely in hemolymph, the invertebrate blood substitute, in contrast to haemoglobin, which is encased in red blood cells. When oxygenated, this pigment gives mollusc and arthropod blood its characteristic blue or purple hue. This review article delves into the fascinating biology of hemocyanin, the copper-based oxygen-carrying protein responsible for "purple blood" in many invertebrates, contrasting its characteristics with the more familiar iron-based hemoglobin. The review used a variety of sources from 2020 to 2025, including preprint sites (bioRxiv, medRxiv), grey literature/press-release outlets including EurekAlert! and ScienceDaily, PubMed, Embase, Scopus, Web of Science, BIOSIS, and Google Scholar. While hemocyanin's unique properties allow for adaptation to diverse environments, its direct application as an artificial human blood substitute faces significant biological and immunological hurdles. The report then transitions to a comprehensive overview of recent advancements in artificial human blood transfusion, focusing on hemoglobin-based oxygen carriers (HBOCs), perfluorocarbon-based oxygen carriers (PFCs), and stem cell-derived red blood cells. This analysis critically examines their development, clinical trial outcomes, and the persistent challenges in achieving safe, effective, and widely available blood alternatives, highlighting the distinct roles and limitations of hemocyanin-derived products primarily in immunomodulation rather than oxygen transport.

**Keywords** Hemocyanin, Extraction and purification of Hemocyanin, Biomedical Applications, Keyhole Limpet Hemocyanin, Clinical trials

## Introduction

Blood is a vital biological fluid, indispensable for maintaining physiological function in complex organisms. Its multifaceted roles encompass oxygen transport, nutrient delivery, waste removal, and immune defense. Acute blood loss, such as that resulting from trauma or surgery, or chronic conditions like anemia, necessitate transfusions to prevent organ shutdown and sustain life. Despite the critical need, traditional blood transfusions are fraught with limitations. A persistent global shortage of donated blood is exacerbated by aging populations, declining donor rates, and disruptions from events such as pandemics. Beyond supply constraints, conventional

DOI: 10.62958/j.cjap.2025.023
www.cjap.ac.cn



Published by CJAP editorial office and Asian BioMed Innovation Press

blood products possess a short shelf life, typically around 42 days, and demand stringent refrigeration, complicating storage and distribution, especially in remote or emergency settings. Furthermore, inherent risks accompany transfusions, including the potential for disease transmission from emerging pathogens (e.g., variant Creutzfeldt-Jakob disease, West Nile Virus, H1N1 virus, hepatitis, HIV) and the induction of immune suppression in recipients [1,2].

The confluence of these challenges—supply limitations, logistical complexities, and safety concerns—underscores a multifaceted crisis in transfusion medicine. This situation has driven an urgent scientific and medical imperative for the development of artificial blood substitutes, more precisely termed "oxygen therapeutic agents" (OTAs). These alternatives are envisioned as universal, pathogen-free, and long-storage solutions, offering particular appeal for emergency medicine, military applications in combat zones, and for patients with specific needs, such as those with rare blood phenotypes or religious objections to traditional transfusions. The demand for artificial blood extends beyond immediate trauma care to include addressing chronic conditions, improving disaster preparedness, and enhancing healthcare delivery in resource-limited environments. This comprehensive need highlights the intricate nature of the problem and the broad benefits a successful substitute could provide [2,3].

Oxygen is a critical molecule for aerobic life, and throughout evolution, animals have developed a wide range of molecular strategies to ensure its efficient transport from the environment to tissues. While hemoglobin is the most widely recognized oxygen-carrying protein, especially in vertebrates, this iron-based pigment is only one of several mechanisms utilized across the animal kingdom. In fact, a number of invertebrates rely on distinctly different molecules, such as hemocyanin, hemerythrin, and chlorocruorin, to meet their oxygen transport needs. These alternative systems often display unique structural, functional, and ecological characteristics, and are particularly suited for environmental niches that hemoglobin may not effectively support. An overemphasis on hemoglobin in scientific literature and education has overshadowed the significance of these non-hemoglobin-based pigments, limiting our appreciation of the full evolutionary spectrum of oxygen transport mechanisms [4,5].

Hemoglobin, a tetrameric protein found within the red blood cells of vertebrates, contains iron at the center of each heme group, allowing it to reversibly bind molecular oxygen. Its cooperative binding behavior—meaning its affinity for oxygen increases as more oxygen molecules are bound—enables efficient oxygen loading in the lungs or gills and unloading in tissues. Hemoglobin is also finely regulated by factors such as pH, carbon dioxide levels, and allosteric effectors like 2,3-BPG, allowing animals to adapt to fluctuating oxygen demands. In humans, it carries approximately 1.34 mL of oxygen per gram, and its cellular encapsulation enhances oxygen delivery and prevents renal filtration loss. While hemoglobin is often hailed for its efficiency, especially in oxygen-rich terrestrial habitats, it is not universally suitable across the wide diversity of ecological systems [6].

In contrast, many invertebrates employ hemocyanin, a copper-based protein that binds oxygen using two copper ions instead of iron. Unlike hemoglobin, which is enclosed in red blood cells, hemocyanin is found dissolved freely in the hemolymph (the invertebrate equivalent of blood). This pigment is responsible for the blue or purple color observed in the blood of mollusks and arthropods when oxygenated. The oxygenated form of hemocyanin, in which copper is oxidized from Cu(I) to Cu(II), exhibits a characteristic blue color. Hemocyanins are structurally massive, often forming multi-subunit complexes ranging from hexamers in arthropods to decamers and even di-decamers in mollusks. These proteins can reach molecular weights exceeding 8 MDa, significantly larger than hemoglobin. While hemocyanin generally exhibits lower oxygen affinity and lacks strong cooperativity, some species, such as horseshoe crabs, have evolved versions with cooperative binding behavior comparable to hemoglobin, indicating functional convergence in certain contexts [7-9].

Hemocyanin is particularly well-suited for cold, low-oxygen environments, such as deep-sea habitats or high-altitude regions. It functions efficiently at low temperatures where hemoglobin might become less effective, making it ideal for ectothermic marine animals like octopuses, squids, and crustaceans. Its free-floating nature in the hemolymph allows for increased diffusion over wider areas, compensating for its relatively lower oxygen-carrying capacity per gram compared to hemoglobin. Furthermore, certain hemocyanins possess multifunctional properties, including roles in immune response, antimicrobial activity, and wound healing, expanding their biological significance beyond mere oxygen transport [5,9].

In addition to hemocyanin, other lesser-known pigments contribute to the diversity of oxygen transport in animals. Hemerythrin, found in sipunculid and priapulid worms, is an iron-based protein that does not contain heme and produces a violet hue

when oxygenated. Chlorocruorin and erythrocruorin, found in various annelids such as marine polychaetes and earthworms, are giant hemoglobin-like proteins with unique structural features that lend them a greenish coloration. Antarctic icefish from the family Channichthyidae present an especially intriguing case, having lost hemoglobin altogether. These fish survive in oxygen-rich but frigid waters by relying on large blood volumes, enhanced cardiac output, and thin-walled capillaries to deliver dissolved oxygen directly via plasma. This diversity underscores the range of adaptations animals have evolved to match oxygen transport systems with environmental demands [10].

Despite this remarkable diversity, the dominance of hemoglobin in biomedical and physiological studies has led to an overly narrow, hemoglobin-centric understanding of oxygen transport. This bias has several limitations. First, it marginalizes the evolutionary relevance of alternative pigments, thereby underappreciating the adaptive significance of hemocyanin and others in numerous phyla. Invertebrates comprise the vast majority of animal species, many of which depend on non-hemoglobin oxygen transport systems, yet these organisms remain underrepresented in physiological research. Second, a hemoglobin-centric framework tends to undervalue environmental adaptations. Hemoglobin is optimized for warm-blooded, terrestrial organisms with stable internal oxygen levels. It cannot fully account for the success of species thriving in cold, hypoxic, or highly variable marine environments, where hemocyanin offers greater functional stability. Third, the structural and functional versatility of hemocyanin remains largely overlooked [11]. Hemocyanins exhibit a broad range of subunit compositions, molecular weights, and functional properties. Some hemocyanins display allosteric regulation, while others are modulated by ions, pH, or temperature. They are also glycosylated, which may play a role in immune recognition or molecular stability. Hemocyanins are actively involved in immune defense, and in some biomedical applications, such as keyhole limpet hemocyanin (KLH), they are used as immune adjuvants or carrier proteins in vaccine development. Ignoring these multifaceted roles results in an incomplete understanding of how respiratory proteins contribute to organismal physiology. Fourth, reliance on oxygen-carrying efficiency per gram as a primary metric is misleading [12]. While hemoglobin is more efficient on a per-mass basis, overall oxygen delivery also depends on physiological traits like blood volume, circulation rate, and metabolic demand. Marine invertebrates may compensate for hemocyanin's lower per-mass efficiency through larger hemolymph volumes or lower metabolic rates, rendering the efficiency gap biologically irrelevant in many cases. Finally, some organisms, such as the hemoglobin-less icefish, challenge the notion that a respiratory pigment is even required for survival, highlighting extreme adaptations that defy conventional models [13].

In light of these limitations, a more holistic perspective is necessary—one that recognizes oxygen transport as a context-dependent, evolutionarily diverse process rather than a one-size-fits-all system dominated by hemoglobin. The present review article aims to illuminate the molecular diversity, ecological relevance, and physiological significance of alternative oxygen carriers. By integrating structural biology, evolutionary biology, and comparative physiology, such a review can reshape our understanding of how animals across the tree of life have solved the universal problem of oxygen transport—often with strategies as colorful and varied as life itself. This review will embark on an exploration of hemocyanin's unique biological attributes and its distinct, albeit specialized, role in biomedical research. This will be critically contrasted with the ongoing efforts and recent advancements in developing hemoglobin-based and other synthetic oxygen carriers for direct human transfusion, clarifying why hemocyanin's primary utility in mammals lies outside direct oxygen delivery.

Between 2020 and 2025, the review drew on multiple sources, including PubMed, Embase, Scopus, Web of Science, BIOSIS, Google Scholar, preprint platforms (bioRxiv, medRxiv), and grey literature/press-release outlets such as EurekAlert! and ScienceDaily. Eligible materials focused on hemocyanin biology, evolution, physiology, or applications, were published in English, and, in the case of press releases, had to be directly linked to a verifiable research source. Items lacking data, unlinked news pieces, and non-English works without translation were excluded. The search strategy, based on both controlled vocabulary and keywords, adhered to PRISMA 2020 and PRISMA-S standards. Two independent reviewers screened all records, with disagreements settled by a third reviewer. Extracted data included bibliographic details, organism studied, methods, and key findings; for press releases, additional details such as issuing organization, publication date, and associated research source were recorded. Findings were synthesized narratively under thematic categories, with all press-release claims verified against their original studies.

## Hemocyanin: The Biology of Purple

## Blood

### Molecular Structure and Oxygen Binding Mechanism

Hemocyanins (Hc) are high molecular weight, copper-containing proteins that function as oxygen carriers in the hemolymph of molluscs and arthropods. The fundamental mechanism of oxygen binding in Hc involves a pair of copper atoms (biscopper sites) directly coordinated by histidine residues from the protein's amino acid side chains. This contrasts with hemoglobin, where iron atoms are embedded within a porphyrin ring structure. In its deoxygenated (deoxy-Hc) state, the copper exists in the cuprous Cu(I) form, rendering the protein colorless. Upon reversible binding of a single dioxygen molecule ($O_2$), the active site undergoes a transformation, forming a Cu(II)-$O_2^{2-}$-Cu(II) complex [14]. This change in oxidation state and coordination imparts the characteristic blue color to the hemolymph. The oxygenated form exhibits distinct absorption bands at 337 nm and 560 nm, which are attributed to charge transfer transitions. Oxygen is bound in a μ-η2:η2 mode, and this binding event induces a conformational change within the active site, notably reducing the copper-copper distance from 4.6 Å in deoxy-Hc to 3.6 Å in oxy-Hc. Hemocyanins are oligomeric proteins, displaying diverse and complex quaternary structures. In arthropods, these proteins can assemble into hexamers, dodecamers, 24-mers, and even 48-mers. For instance, Limulus hemocyanin, a 48-mer, possesses a molecular weight of approximately 3,600,000 Da. Molluscan hemocyanins are generally even larger, forming giant cylindrical molecules that can reach molecular weights of up to 9,000,000 Da, composed of approximately 20 polypeptide chains. Each arthropod Hc subunit typically weighs about 75 kilodaltons (kDa) and contains a single oxygen-binding active site [15-17].

Hemocyanin and hemoglobin are both oxygen-transport proteins but differ fundamentally in their metal centers and structural architecture. Hemoglobin contains iron ($Fe^{2+}$) at the core of its heme group, which binds oxygen reversibly. It is a tetrameric, globular protein found in red blood cells of vertebrates, giving blood its red color upon oxygenation. In contrast, hemocyanin utilizes copper ($Cu^{+}$) ions directly coordinated by histidine residues, without a heme group. Oxygen binds between two copper atoms in the active site, forming a dioxygen complex that turns the blood blue upon oxygenation. Hemocyanins are typically found freely dissolved in the hemolymph of mollusks and arthropods and often exist as large oligomeric complexes, forming multi-subunit cylindrical or hexameric structures [16].

This copper-based mechanism represents an evolutionary adaptation to different environmental and physiological needs. Simplified structural diagram comparing hemoglobin (iron-based) and hemocyanin (copper-based) shown in Figure 1:

- Hemoglobin: Tetramer with 4 subunits, each containing an iron ($Fe^{2+}$) in a heme group that binds oxygen.
- Hemocyanin: Often hexameric or larger oligomers,

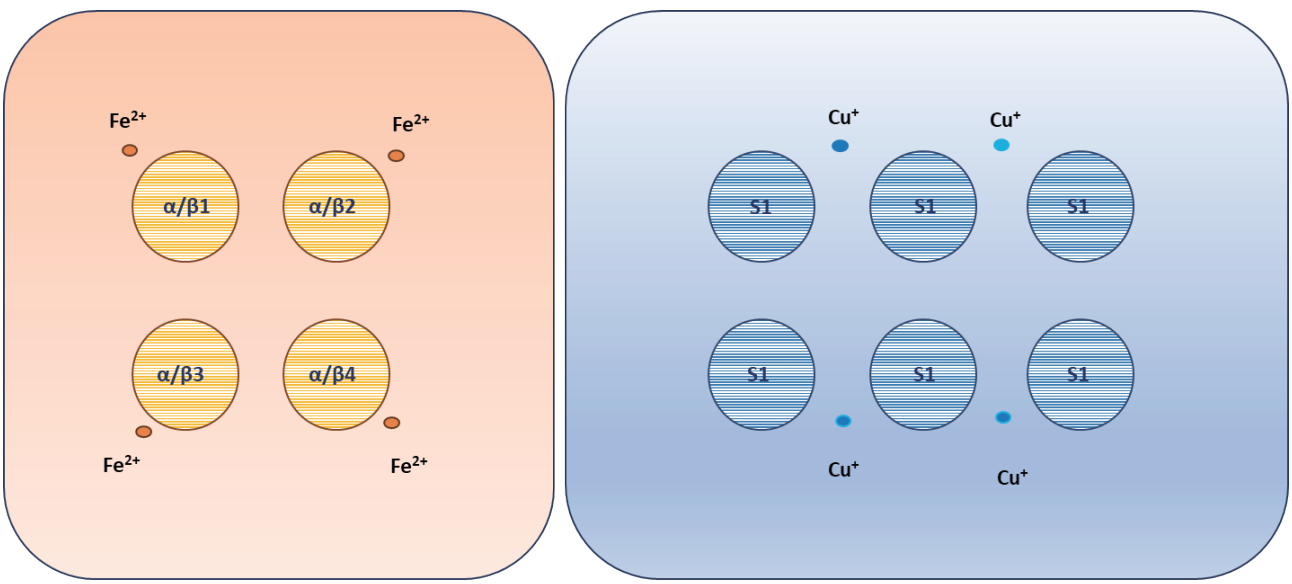


**Figure 1:** Structural composition of hemoglobin and hemocyanin

with pairs of copper ($Cu^+$) ions between subunits binding oxygen directly.

**Molecular Size:** Hemocyanins are exceptionally large macromolecules, with molluscan Hc reaching up to 9 MDa and arthropod Hc up to 3.6 MDa. They are typically found free-floating in the hemolymph of invertebrates. While large size contributes to hemocyanin's immunogenicity, it also poses distinct physiological challenges in a mammalian closed circulatory system. Unlike hemoglobin, which is compartmentalized within red blood cells, free-floating large proteins can significantly increase colloidal osmotic pressure, potentially leading to fluid shifts and edema. Furthermore, while too-small HBOCs have been observed to leak out of vessels, excessively large molecules might also face issues with distribution, viscosity, or clearance mechanisms not adapted for such free-floating giants. The large molecular size, combined with immunogenicity and suboptimal oxygen affinity, presents multiple, compounding biological hurdles that collectively render hemocyanin impractical for direct human blood transfusion [17,18].

## Allosteric Regulation and Physiological Adaptations in Invertebrates

The respiratory functions of hemocyanins are characterized by a wide range of allosteric properties, enabling organisms that possess these large molecules to adapt precisely to their varied environmental conditions. Oxygen binding by Hc is finely regulated by various allosteric effectors, including hydrogen ions (pH), l-lactate, urate, and inorganic ions such as chloride, calcium, and magnesium. A decrease in pH, signifying an increase in H+ concentration, typically leads to a reduction in oxygen affinity and cooperativity, a phenomenon functionally analogous to the Bohr effect observed in hemoglobin. Conversely, an increase in l-lactate concentration generally enhances both oxygen affinity and the cooperativity of binding. Cooperative oxygen binding, evidenced by a sigmoid oxygen binding curve and Hill coefficients greater than 1 (e.g., 1.6–3.0 in certain arthropod species like horseshoe crabs), is commonly observed in larger hemocyanin molecules. This cooperativity can be influenced by the specific arrangement of subunits within larger protein complexes [19,20].

The oxygen-binding profile of hemocyanin is also significantly influenced by temperature. At lower temperatures, cooperativity tends to increase with pH, whereas at higher temperatures, cooperativity appears to be less dependent on pH. These adaptive properties are particularly evident in the ecological distribution of species that utilize hemocyanin. For example, crustaceans inhabiting cold environments with low oxygen pressure are well-suited to hemocyanin-based oxygen transport, as it demonstrates superior efficiency under these specific conditions compared to hemoglobin. The intricate allosteric regulation and optimized performance of hemocyanin under specific environmental conditions, such as cold temperatures and low oxygen availability, point to a highly specialized evolutionary trajectory [21]. This contrasts with the broader functional range of hemoglobin, which supports the higher metabolic rates and thermoregulation characteristic of large, warm-blooded terrestrial and aquatic vertebrates. The observation that organisms utilizing hemocyanin do not require hemoglobin, and conversely, hemoglobin-dependent organisms could not survive with hemocyanin, underscores this fundamental specialization. The complex interplay of pH, temperature, and ionic cofactors in fine-tuning hemocyanin's oxygen affinity is a testament to its precise adaptation to niche environments. This deep-seated environmental and physiological specialization implies that hemocyanin, in its native form, is fundamentally ill-suited for direct oxygen transport within the mammalian physiological environment. Its optimal operating parameters are incongruent with the high oxygen demands and stable warm temperatures of human blood, making direct transplantation functionally impractical without radical structural and functional re-engineering [11, 17].

## Hemocyanin vs. Hemoglobin: A Comparative Analysis of Efficiency and Evolutionary Trajectories [22-26]

### Structural and Cellular Context Differences

Hemoglobin contains iron atoms housed within porphyrin (heme) rings, which reversibly bind oxygen. In vertebrates, hemoglobin is tightly packaged within red blood cells, a cellular compartmentalization that ensures its stability against proteolysis, maintains a low colloid osmotic pressure in the blood, and prevents its rapid filtration and loss by the kidneys.14 In contrast, hemocyanin utilizes copper atoms directly coordinated by histidine residues for oxygen binding, rather than a heme prosthetic group. Hemocyanin circulates freely dissolved in the hemolymph, not confined within cells. This free-floating nature may offer advantages for navigating very narrow vessels within the open circulatory systems characteristic of

many invertebrates.

### Oxygen Binding and Transport Efficiency

Hemocyanin generally exhibits a significantly lower oxygen transport capacity compared to human hemoglobin, being approximately one-fourth as efficient per unit of blood. Most hemocyanins bind oxygen non-cooperatively, meaning that the binding of oxygen at one active site does not significantly enhance the affinity of other binding sites. This non-cooperative binding is suboptimal for the rapid and efficient oxygen loading and unloading required in high-metabolism systems. In stark contrast, hemoglobin displays cooperative oxygen binding, a hallmark of its efficiency. The binding of the first oxygen molecule to a hemoglobin subunit induces conformational changes, including a 15° rotation of subunits from a tense (T) deoxygenated state to a relaxed (R) oxygenated state. This allosteric transition increases the affinity of subsequent binding sites, leading to highly efficient oxygen loading in the lungs and effective unloading in metabolically active tissues.16 However, it is important to acknowledge that in specific ecological niches, such as cold, low-oxygen aquatic environments, hemocyanins can be more efficient oxygen transporters than hemoglobin, demonstrating their specialized adaptation.

### Physiological and Evolutionary Context

Hemoglobin evolved to meet the high oxygen demands of larger, warm-blooded (endothermic) and terrestrial organisms. These organisms require substantial oxygen for thermoregulation and efficient transport over long distances within their complex closed circulatory systems.16 Hemocyanin, conversely, is perfectly adequate for smaller, cold-blooded (ectothermic) creatures with lower metabolic rates and shorter oxygen transport distances, often living in aquatic or cold environments. From an evolutionary perspective, hemocyanin is considered an older characteristic, with hemoglobin emerging later to facilitate the transition to new environments and lifestyles that demanded higher oxygen flux.

### Carbon Monoxide Affinity

A notable distinction lies in arthropod hemocyanin's low affinity for carbon monoxide (CO), which presents a significant advantage over hemoglobin. Hemoglobin binds CO with much higher affinity than oxygen, forming carboxyhemoglobin, which is why CO poisoning is dangerous and often lethal in vertebrates. This suggests that arthropods may possess a considerable degree of resistance to CO poisoning.

The comparative analysis reveals not merely differences in structure and efficiency, but fundamental, almost irreconcilable, physiological mismatches between hemocyanin and human hemoglobin (Table 1). Hemoglobin's highly efficient, cooperative oxygen binding and its cellular encapsulation are critical adaptations for the high metabolic demands, warm body temperature, and complex closed circulatory system of mammals. Hemocyanin's lower efficiency, often non-cooperative binding, and free-floating nature, while perfectly suited for its native cold, low-oxygen, and open circulatory environments, are directly counter to human physiological requirements. The evolutionary divergence underscores that these are optimized solutions for distinct biological problems. This deep-seated functional and evolutionary incompatibility strongly suggests that hemocyanin is not a viable direct oxygen carrier for human blood transfusion. Its inherent properties would lead to insufficient oxygen delivery and potential systemic issues under mammalian physiological conditions, regardless of other modifications. This understanding is crucial for appreciating why research has not pursued hemocyanin as a primary oxygen-carrying blood substitute [17, 19, 24].

## Sources and Extraction of Hemocyanin

Hemocyanin is most commonly sourced from marine mollusks and arthropods, especially Megathura crenulata (keyhole limpet) and penaeid shrimp such as *Litopenaeus vannamei* and *Macrobrachium acanthurus*. The giant keyhole limpet yields Keyhole Limpet Hemocyanin (KLH), a glycosylated didecameric copper containing metalloprotein (~8–32 MDa, ~3,400 amino acids per monomer) prized for its immunogenicity and biomedical utility. Shrimp hemocyanins (e.g. from *L. vannamei*) offer a lower molecular weight (~72–76 kDa per subunit), more soluble variant that is amenable to purification from large-scale aquaculture operations [26].

Extraction begins with collection of hemolymph. Shrimp hemolymph is typically drawn from the pericardial sinus under cold anesthesia and anticoagulated (e.g., sodium citrate), followed by low speed centrifugation (~3,000 g) to remove cellular debris. Limpet hemolymph, harvested either by lethal cardiac incision or non lethal methods (if applicable), is clarified similarly. The cell free supernatant then

**Table 1:** Hemoglobin vs Hemocyanin

| Property | Hemoglobin | Hemocyanin |
|---|---|---|
| Primary Function | Oxygen transport in vertebrates | Oxygen transport in invertebrates (arthropods, mollusks) |
| Metal Center | Iron ($Fe^{2+}$) | Copper ($Cu^{+}$) |
| Color (Oxygenated) | Bright red | Blue |
| Color (Deoxygenated) | Dark red | Colorless or grayish |
| Oxygen Binding Site | Central $Fe^{2+}$ atom in heme group | Between two $Cu^{+}$ ions coordinated by histidine residues |
| Oxygen Binding Mechanism | Reversible binding without oxidation | Reversible, with temporary oxidation of $Cu^{+}$ to $Cu^{2+}$ |
| Coordination Complex | $Fe^{2+}$ coordinated in porphyrin ring (heme) | $Cu^{+}$ coordinated by 3 histidine ligands each |
| Molecular Formula (active site) | $C_{34}H_{32}FeN_4O_4$ (heme) | $[Cu_2O_2(His)_6]$ complex |
| Quaternary Structure | Tetramer ($\alpha_2\beta_2$ subunits) | Hexamers to didecamers (12–48 subunits), huge complexes |
| Molecular Weight | ~64 kDa | 1.5–9 MDa (depending on species) |
| Solubility | Packed inside red blood cells | Freely dissolved in hemolymph |
| Occurrence | Vertebrates, some annelids and insects | Arthropods (crabs, spiders), mollusks (octopus, snails) |
| Affinity to $O_2$ | Moderate; regulated by pH, $CO_2$ (Bohr effect) | Lower affinity; temperature and ions affect binding |
| $O_2$ Carrying Capacity | ~1.34 mL $O_2$/g of Hb | ~0.4 mL $O_2$/g of hemocyanin |
| Immune Role | None | Some antimicrobial activity observed in invertebrates |
| Buffering Role | Yes, acts as a pH buffer via histidine residues | Minimal buffering capacity |
| Shelf Life (in vitro) | ~35–42 days (refrigerated blood storage) | Often more stable in solution, up to several months refrigerated |
| Thermal Stability | Denatures >42°C | Higher stability in extreme temps for some species |
| Evolutionary Insight | Adapted for efficient $O_2$ transport at high metabolic rates | Evolved in marine/low-oxygen environments for slower metabolism |
| Coloration Use | Diagnostic (oxygenation levels) | Blue blood sometimes used in research (e.g., Limulus) |
| Medical Use | Blood transfusions, artificial blood research | Hemocyanin from horseshoe crab used in endotoxin testing (LAL test) |
| Toxicity (Free form) | Free hemoglobin is toxic to kidneys | Hemocyanin less toxic in free form |

undergoes fractionation and purification, most often using ammonium sulfate precipitation and stepwise dialysis for concentration and desalting. Further purification is achieved using gel filtration (size exclusion) chromatography (e.g., Sephadex G 100 or Superdex columns) to isolate high molecular weight oligomeric hemocyanin complexes. Additional polishing steps employ ion exchange chromatography to separate isoforms and remove contaminants [27].

In shrimp hemocyanin extraction workflows, ultracentrifugation (~250,000 g, 4 °C) helps isolate the hexameric protein fraction (~450 kDa), which is then redissolved in buffered solution (e.g., Tris HCl with $Ca^{2+}$, pH 7.0) and quantified spectroscopically (e.g., $\varepsilon_{278} = 1.1$ (mg/mL)·$cm^{-1}$) (Figure 2) [28]. Throughout purification, care is taken to preserve copper coordination and glycosylation, since deglycosylation disrupts quaternary structure and diminishes immunogenic properties as evidenced in KLH, *Fissurella latimarginata*, and *Concholepas hemocyanins*.

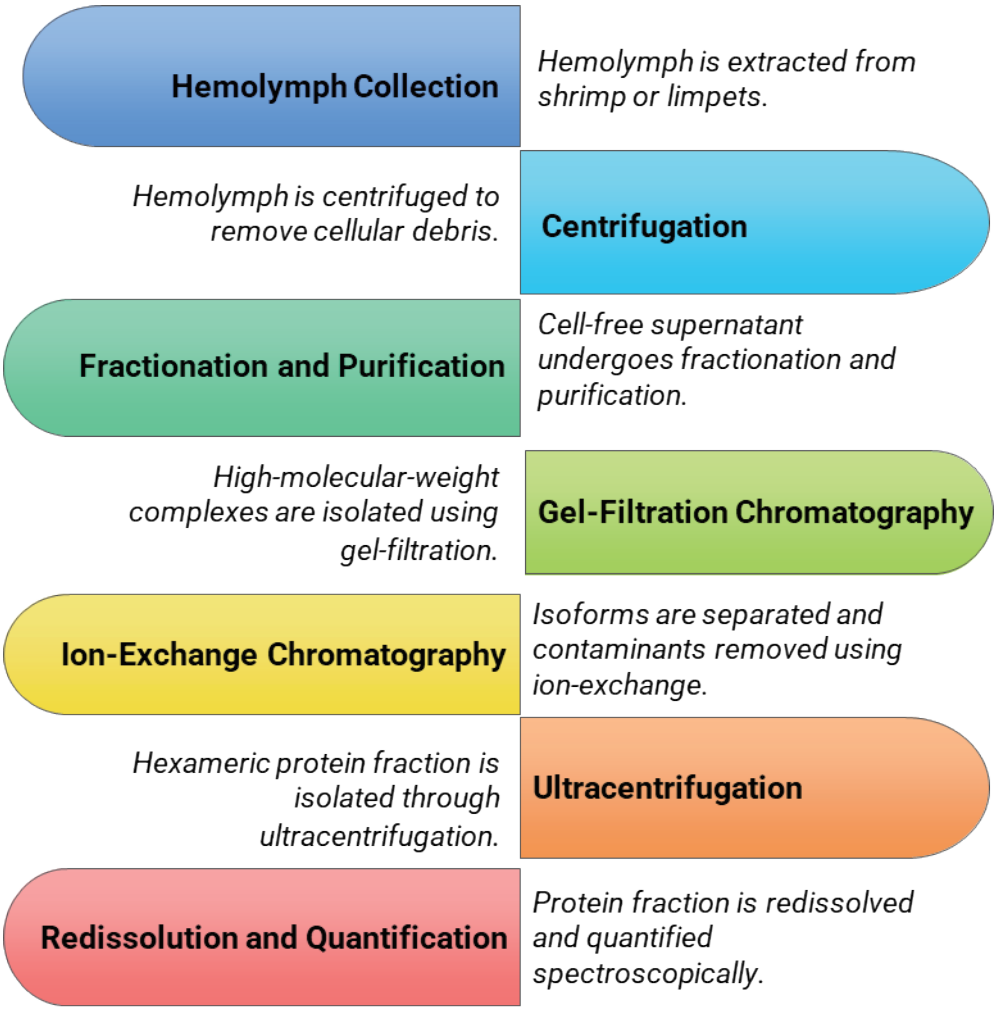


**Figure 2:** Extraction and purification of hemocyanin

## Multifunctional Roles of hemocyanins: Beyond Oxygen Transport

Beyond their primary role in oxygen transport, hemocyanins are increasingly recognized for their diverse multifunctional roles in arthropods and molluscs. These functions include crucial involvement in immune defense and metabolic regulation [17]. These proteins exhibit significant immunostimulatory properties, including documented antiviral and phenoloxidase-like activities [29]. Hemocyanin shares structural homology with phenol oxidases (e.g., tyrosinase), both belonging to the family of type-3 copper proteins and possessing similar copper-binding coordination centers. Hemocyanin itself can exhibit phenol oxidase activity, which can even be enhanced by partial denaturation, suggesting a latent enzymatic potential within its structure.

Molluscan hemocyanins, in particular, are potent immunostimulants in mammals. This property is strongly associated with their exceptionally large molecular size, intricate quaternary structure, xenogeneic character, and unique glycosylation patterns (up to 9% w/w, with mannose being a predominant carbohydrate). The observation that hemocyanin's oxygen-carrying capacity is suboptimal for human physiology, while its distinct immunogenic properties in mammals are highly valuable, reveals a fascinating dichotomy. The very characteristics that render it an inefficient oxygen carrier (large size, foreign nature, complex glycosylation) are precisely what make it a potent immunostimulant [30]. This represents a biological molecule being effectively repurposed for a therapeutic function entirely different from its primary role in its native organism, demonstrating an unexpected translational utility. This distinct application of hemocyanin in biomedicine, leveraging its immune-stimulating capabilities, is paramount for understanding its actual relevance in the broader context of artificial blood research.

## Hemocyanin in Biomedical Applications:

## Immunomodulation, Not Direct Transfusion

### Keyhole Limpet Hemocyanin (KLH) as a Vaccine Carrier and Immunoadjuvant in Cancer Therapy

Keyhole Limpet Hemocyanin (KLH), derived from the gastropod *Megathura crenulata*, has been extensively utilized for decades in various immunological and clinical applications, primarily as a potent immunomodulator. KLH functions as a natural, nontoxic, nonpathogenic, and nonspecific immunostimulant, notably demonstrating efficacy in the treatment of superficial bladder cancer. Its primary application in vaccine development is as a carrier protein for haptens—small molecules, peptides, or drug molecules that are not immunogenic on their own (Table 2). By chemically conjugating these haptens to KLH, a robust immune response and subsequent antibody production can be stimulated in mammals. KLH's exceptional immunogenicity in mammals is attributed to several key features: its xenogeneic character (phylogenetically distant from mammalian proteins, thus recognized as foreign), its immense size (ranging from approximately 4 to 8 MDa), its intricate quaternary structure, and its complex, heterogeneous glycosylation patterns (up to 9% w/w, with mannose being a predominant carbohydrate) [18, 25, 30].

Preclinical studies and early-phase clinical trials are actively investigating KLH as an adjuvant in therapeutic cancer vaccines. This involves conjugating specific tumor-associated antigens to KLH to stimulate targeted anti-tumor immune responses. Examples include studies in oral squamous cell carcinoma, breast cancer, prostate cancer, and melanoma. Research indicates that KLH induces a robust, specific humoral response, predominantly characterized by IgG2a antibodies, and a sustained cellular response, manifesting as a delayed hypersensitivity reaction. Furthermore, KLH activates and matures human dendritic cells, a process partially mediated by the mannose receptor, which recognizes mannose and fucose ligands present on KLH. The consistent observation that KLH's utility in biomedical applications is directly attributable to its high immunogenicity in mammals creates a striking paradox. The very property that makes it highly effective for immunotherapy (eliciting a strong immune response) is the primary reason it is unsuitable for direct oxygen-carrying blood transfusion, where immune compatibility is paramount. This highlights a nuanced understanding of biological function and its context-dependent utility. This understanding is critical for the report's central theme, firmly establishing that while hemocyanin has significant clinical relevance, it is precisely for applications that leverage its "foreignness" and immune-stimulating capabilities, rather than attempting to suppress them for oxygen transport [27-31].

### Challenges Limiting Hemocyanin's Direct Use as an Oxygen-Carrying Blood Substitute in Humans

Despite its role as an oxygen carrier in invertebrates, several fundamental biological and physiological challenges preclude hemocyanin's direct use as an oxygen-carrying blood substitute in humans.

#### *Immunogenicity*

Humans do not naturally produce hemocyanin, and its xenogeneic nature ensures it is profoundly recognized as an "alien protein" by the human immune system. Direct transfusion of hemocyanin would likely trigger a severe, potentially fatal, immune reaction akin to an incompatible blood transfusion, involving a violent systemic response. The large molecular weight, structural heterogeneity, and complex glycosylation patterns of hemocyanin are precisely what make it one of the strongest antigens known in mammals, leading to powerful antiserum formation and macrophage invasion at the site of application. While theoretical strategies like encapsulation within liposomes or PEGylation might attempt to mask its immunogenicity, these approaches are highly speculative and have not been demonstrated for hemocyanin as an oxygen carrier in the context of blood substitution. The human immune system is exquisitely designed to detect and eliminate foreign invaders. Hemocyanin, being phylogenetically distant and possessing highly immunogenic characteristics (large size, complex structure, xenogeneic origin), would be immediately recognized as non-self [26]. The severity of immune reactions to incompatible blood types, often due to carbohydrate antigens, suggests that a complex foreign protein like hemocyanin would elicit an even more profound and dangerous response. This is not a minor side effect but a core biological incompatibility. This barrier is so fundamental and severe that it effectively renders hemocyanin non-viable as a direct oxygen-carrying blood substitute for human transfusion. The risk of life-threatening immune rejection outweighs any potential oxygen-carrying benefit, making extensive research into this specific application largely futile without a revolutionary method to completely bypass or re-engineer human immune recognition [4, 19, 31].

#### *Oxygen Affinity and Efficiency*

Most hemocyanins are significantly less efficient at

**Table 2:** Immunological Applications of KLH

| Feature/Parameter | KLH as Vaccine Carrier | KLH as Immune Stimulant |
|---|---|---|
| Primary Role | Conjugates to weak or non-immunogenic antigens to enhance adaptive immune response | Directly stimulates innate immune pathways and acts as a non-specific adjuvant |
| Mechanism of Action | Presents multiple T-helper epitopes, facilitates antigen uptake by APCs, and enhances MHC-II presentation | Activates NF-κB pathway via Syk and ERK phosphorylation; induces cytokine secretion (e.g., IL-6, TNF-α) |
| Antigens Commonly Conjugated | Peptides, haptens (e.g., DNP), tumor-associated carbohydrates (Globo H, MUC1, GD3), and idiotype antigens | N/A (used alone to trigger immune activation) |
| Type of Immune Response Induced | T-dependent B-cell responses, class switching (IgM → IgG), Th1/Th2 skewing based on formulation | Inflammatory cytokines, monocyte/macrophage activation, neutrophil recruitment, non-antigen-specific activation |
| Clinical Applications | Therapeutic cancer vaccines (melanoma, prostate, breast, bladder); preclinical immunogenicity studies | Used in challenge models for evaluating immune modulation (e.g., KLH skin tests or serum antibody measurement) |
| Dosage Range in Studies | 100–2500 µg per injection (subcutaneous or intradermal) | Similar range; varies by study; given alone or with adjuvants (e.g., Montanide, alum) |
| Formulations | Conjugated to antigen using glutaraldehyde, maleimide, or EDC chemistry | Administered in buffered saline or with immune enhancers |
| Clinical Trials | Globo H–KLH, BEC2–KLH (GD3 mimic), MUC1–KLH in various Phase I–III trials | Widely used in immunotoxicology, vaccine evaluation, and pharmacodynamic testing of immune-modulating drugs |
| Immunological Readouts | Anti-KLH IgG, IgM titers, T-cell proliferation, cytokine profiling (IFN-γ, IL-2) | Cytokine release (IL-6, IL-1β, TNF-α), flow cytometry of monocyte activation markers (CD80, CD86) |
| Duration of Response | KLH induces long-lasting humoral responses; memory response on re-exposure | Innate effects are rapid (hours to days); can potentiate adaptive responses when co-administered |
| Animal Models Used | Mice, rats, guinea pigs, rabbits, non-human primates | THP-1 monocyte line, mice, guinea pigs; often used in LAL alternatives |
| Adjuvant Compatibility | Compatible with alum, Montanide, MPLA, QS-21 | Functions with or without adjuvants; effect enhanced with cytokines or toll-like receptor agonists |
| Advantages | Strong and reproducible immune enhancement; universal carrier protein; minimal toxicity | Strong innate activation; applicable in preclinical safety and efficacy testing |
| Limitations | Large size can cause aggregation; conjugation chemistry must be optimized for stability | May cause transient inflammation; batch variability; not suitable for all patients (rare hypersensitivity) |
| Storage and Stability | Stable in lyophilized form; reconstituted KLH requires refrigeration; sensitive to pH and aggregation | Same as vaccine form; long shelf life if kept desiccated and cold |
| Commercial Forms Available | KLH (Crude), KLH (Subunit), c-KLH (carrier-grade), Conjugated KLH products | Same variants; typically unmodified or combined in immune testing kits |
| Regulatory Status | GRAS for research use; used in FDA-registered clinical trials (e.g., cancer vaccines, allergy vaccines) | Approved in challenge models; standard antigen for immunocompetence testing in toxicology and immunotherapy trials |

transporting oxygen compared to human hemoglobin, being roughly one-fourth as effective per amount of blood. They often exhibit non-cooperative oxygen binding, which is suboptimal for efficient oxygen loading in the lungs and unloading in the tissues under mammalian physiological conditions. The high oxygen demand of the mammalian warm-blooded metabolism—a "warm furnace" that "demands much oxygen, always"—could not be met with hemocyanin, which is better suited for colder, less oxygen-rich environments. Mammalian physiology is characterized by high metabolic rates, large body sizes, and endothermy, all of which necessitate a highly efficient and responsive oxygen transport system. Hemocyanin's lower oxygen capacity and often non-cooperative binding, while adapted for its native environment, would simply be insufficient to sustain human cellular respiration and organ function. The "stickiness" of oxygen to hemocyanin in some contexts further implies a difficulty in releasing oxygen to needy tissues, which is critical for a functional oxygen carrier. Therefore, even if the immunological challenges could be overcome, hemocyanin's inherent functional limitations under human physiological conditions (temperature, oxygen partial pressures, metabolic demand) make it fundamentally unsuitable as a primary oxygen carrier for human blood [29, 30].

## Recent Advances in Artificial Human Blood Transfusion: Current Oxygen Carrier Technologies

The concept of "purple blood" as artificial human blood transfusion refers to the distinctive color observed in some advanced artificial blood substitutes, particularly those utilizing processed hemoglobin. This color arises because the hemoglobin, when extracted and encased, doesn't oxidize in the same way as in natural red blood cells until it's actually used, resulting in a purplish hue. The period from 2020 to 2025 has seen significant strides, especially in Japan, in the development and initial clinical trials of these next-generation artificial blood products [31, 32].

The pursuit of artificial human blood has gained remarkable momentum between 2020 and 2025, with a particular focus on "purple blood" due to its unique composition and potential as a universal transfusion alternative. This distinctive coloration arises from the way hemoglobin is processed and encapsulated in these advanced blood substitutes; unlike natural red blood cells where hemoglobin is consistently oxygenated, the hemoglobin in these artificial formulations doesn't undergo the same oxidation until it's introduced into the body, leading to a purplish hue. This period has seen significant breakthroughs, predominantly emanating from Japan, in the development and initial human clinical trials of these next-generation artificial blood products, specifically hemoglobin vesicles (HbVs) [33].

A major advancement lies in the sophisticated design of HbVs, which encapsulate purified and highly concentrated hemoglobin within protective phospholipid vesicles, effectively mimicking the cellular structure of natural red blood cells. This design is crucial for mitigating the adverse effects, such as vasoconstriction, that plagued earlier acellular hemoglobin-based oxygen carriers (HBOCs) by preventing the free hemoglobin from directly scavenging nitric oxide in the bloodstream. Notably, the hemoglobin for these HbVs is often sourced from *expired donated blood*, a revolutionary approach that transforms medical waste into a life-saving resource [34]. This universal compatibility, being blood-type-free and pathogen-free by design, is a game-changer, eliminating the critical need for cross-matching in emergency situations. Furthermore, the impressive shelf life of up to two years at room temperature, and potentially longer under refrigeration, for these artificial blood products dramatically outperforms the mere month-long viability of traditional donated blood, offering unparalleled logistical advantages for disaster relief, military operations, and remote medical care. Preclinical studies, primarily in animal models, have consistently demonstrated the efficacy of HbVs in maintaining oxygen transport and stabilizing blood pressure even in severe hemorrhagic shock and stroke [35].

The clinical trial landscape from 2020 to 2025 has been characterized by cautious but determined progress, with Japan at the forefront. Following promising small-scale studies conducted as early as 2022, which confirmed the safety and oxygen-carrying capacity of HbVs in human volunteers, a significant milestone is the planned commencement of formal Phase I clinical trials by March 2025 in Japan [36, 37]. These trials, led by institutions like Nara Medical University, involve administering 100 to 400 milliliters of the artificial blood to healthy adult volunteers, with the primary objective of rigorously assessing its safety and tolerability. Should these trials prove successful, the ambitious target is to make this "purple blood" technology available for widespread clinical use by 2030, potentially positioning Japan as the first nation to implement such a transformative medical innovation [37-43]. While Japan leads in HbV trials, other avenues of artificial blood research are also progressing globally; for instance, the UK saw clinical

trials in 2022 involving the transfusion of laboratory-grown red blood cells derived from stem cells, and the US Defense Advanced Research Projects Agency (DARPA) continues to fund synthetic blood research for military applications, with human trials anticipated in the later part of the decade. Despite the immense promise, challenges persist, including ensuring long-term safety and efficacy in larger populations, scaling up cost-effective production, and ultimately developing products that can fully replicate the myriad functions of natural blood beyond just oxygen transport, such as clotting and immune response. Nevertheless, the advancements in "purple blood" represent a compelling leap toward addressing global blood shortages and revolutionizing emergency and critical care medicine [44].

## Recent Advances (2020-2025)

**Hemoglobin Vesicles (HbVs):** The primary focus of recent advancements has been on hemoglobin vesicles (HbVs). These are sophisticated artificial blood cells that encapsulate purified and concentrated hemoglobin (the oxygen-carrying molecule) within protective phospholipid vesicles (liposomes). This cellular-structured approach aims to mitigate the risks associated with earlier generations of acellular hemoglobin-based oxygen carriers (HBOCs), which sometimes caused adverse effects like vasoconstriction due to direct nitric oxide scavenging. A notable advance is the ability to extract hemoglobin from expired donated blood. This re-utilizes a resource that would otherwise be discarded, addressing issues of blood shortage and waste [45].

A key breakthrough is that these artificial cells are "blood-type-free" and pathogen-free. By removing the original membrane that determines blood types, they eliminate the need for cross-matching, making them universally compatible. This is a crucial advantage in emergency situations where immediate transfusion is needed without time for blood typing. Unlike traditional donated blood which has a short shelf life (around a month under refrigeration), these artificial blood products can be stored for significantly longer. Research indicates a shelf life of up to two years at room temperature, and even longer (up to five years) under refrigeration. This dramatically improves logistical challenges, especially in disaster zones or remote areas. Studies, primarily in animal models, have demonstrated the efficacy of HbVs in carrying oxygen throughout the body and stabilizing blood pressure, even in cases of massive hemorrhage and stroke. As mentioned, the characteristic "purple blood" color is a result of the processed hemoglobin within the artificial cells, distinguishing them visually from natural red blood.

Hemoglobin-based oxygen carriers (HBOCs) represent the most extensively researched and commercially advanced segment of artificial blood substitutes, utilizing modified hemoglobin to transport oxygen throughout the body. HBOCs offer several significant advantages over traditional blood, including universal compatibility (no blood typing or cross-matching required), pathogen-free status, and the potential for long-term storage (some up to three years) at room temperature, eliminating the need for refrigeration [37-46]. These properties make them immediately available for emergency situations, such as in military or trauma scenarios (Table 3).

**Challenges and Toxicity:** Despite their promise, early generations of acellular, chemically modified HBOCs (e.g., diaspirin cross-linked Hb (DCLHb)

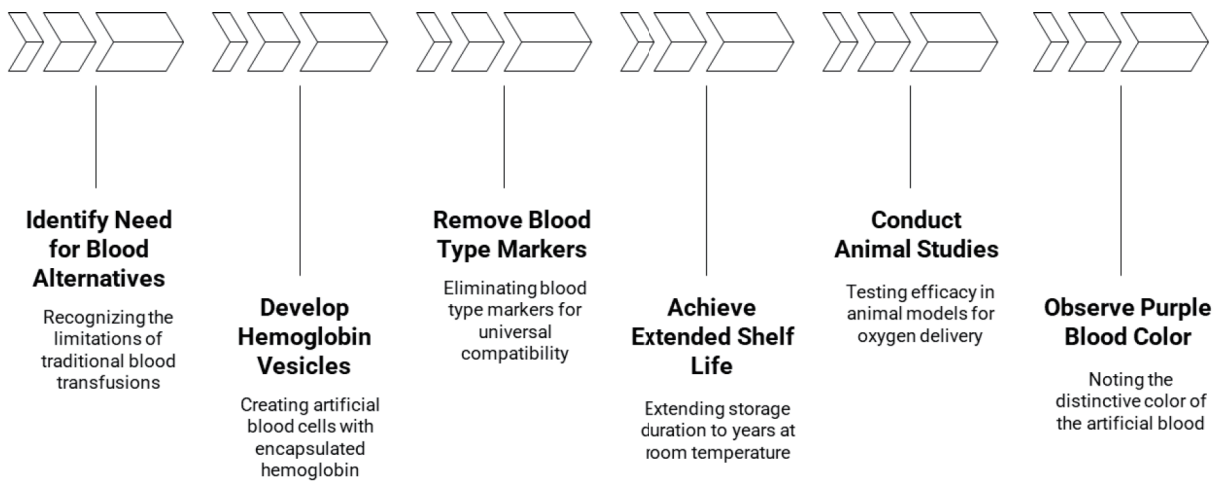


**Figure 3:** Development and use of hemoglobin vesicles

**Table 3:** Classification, Key Characteristics, and Clinical Status of Hemoglobin-Based Oxygen Carriers (HBOCs)

| HBOC Type/ Class | Specific Product Name | Source | Key Modification/ Design | Key Advantages | Key Disadvantages/ Toxicity | Clinical Status/ Regulatory Approval |
|---|---|---|---|---|---|---|
| First-generation | HemAssist (DCLHb) | Human Hb | Diaspirin cross-linked | Reduces tetramer dissociation, low autoxidation rates 13 | High NO scavenging, vasoconstriction, hypertension, cardiac toxicity, increased mortality, renal damage | Discontinued (1999) |
| First-generation | Optro | Recombinant Hb | Cross-linked tetramer | Reduced dissociation | Failed Phase 2 | Discontinued |
| Second-generation | PolyHeme | Human Hb | Polymerized | Less heme loss than cross-linked | Higher mortality rates, serious adverse events | Discontinued |
| Second-generation | Hemopure (HBOC-201) | Bovine Hb | Polymerized | Clinical efficacy in severe anemia, room temperature storage (up to 3 years), pathogen-free, universal compatibility | Temporary skin discoloration, elevation of blood pressure, increased enzymes (troponin) | Approved for acute anemia in South Africa; Expanded access programs in U.S. |
| Second-generation | Oxyvita2 | Bovine Hb | Polymerized | Lyophilized for easy storage/ transport 13 | (Not specified in provided text) | (Not specified in provided text) |
| Second-generation | Oxyglobin | Bovine Hb | Polymerized | (Veterinary use) | (Veterinary use) | Veterinary product |
| Third-generation | Hemospan | Human Hb | PEGylated | Less vasoconstriction | Increased heme loss, failed Phase 3 clinical trials | Discontinued |
| Third-generation | Sanguinate | PEGylated Bovine Hb | Conjugated | Less vasoconstriction | Increased heme loss | (Not specified in provided text) |
| New-generation | ErythroMer | Human Hb | Encapsulated in artificial membrane, pH-responsive | Mimics RBC size/ pH-responsive O2 affinity, 2-3x more potent than stored RBCs, dried powder, 1+ year shelf life, easy transport | Clinical studies not completed | Preclinical trials in rabbits, human trials finalizing |
| New-generation | Hemoglobin Vesicles (HbVs) | Human Hb | Encapsulated in PEGylated phospholipid vesicles | Shields toxic effects of molecular Hb | Mild to moderate fever | Phase 1 clinical trial (Japan, 2020) |
| New-generation | HEMO2life (M101) | Marine Worm Hb | Naturally Polymerized (extracellular) | Efficacy in organ preservation (kidney), no vasoactive side effects | Further human trials needed for intravenous use safety | Approved for ex-vivo kidney perfusion in EU |

like HemAssist, and polymerized human Hb like PolyHeme) encountered significant safety issues in clinical trials. These included high rates of nitric oxide (NO) scavenging, leading to severe vasoconstriction and hypertension, myocardial infarction, kidney damage, and, critically, increased mortality rates in patient cohorts. This toxicity is primarily attributed to free hemoglobin molecules, which, when outside the protective environment of red blood cells, undergo disruptive and irreversible oxidation. This reaction renders the HBOC highly chemically reactive, contributing to the observed adverse cardiovascular and renal complications. Free Hb can also extravasate from blood vessels, leading to issues like temporary skin discoloration.2 The optimal oxygen affinity (p50) for HBOCs is still a subject of ongoing research. While earlier products targeted p50s close to fresh human blood, newer conjugated and encapsulated HBOCs have explored both higher and lower affinities, with some, like ErythroMer, designed to be pH-responsive for dynamic oxygen offloading [47, 48].

**Newer Generations and Clinical Status:**

- **Second-generation (Polymerized Hb):** Hemopure (HBOC-201), derived from bovine hemoglobin, has demonstrated clinical efficacy in expanded access programs for severe anemia in the U.S. and is approved for acute anemia in South Africa. Its stability allows for room temperature storage for up to three years. However, another polymerized product, PolyHeme, was associated with higher mortality rates and serious adverse events in clinical trials and was subsequently discontinued [49].
- **Third-generation (Conjugated Hb):** Products like Hemospan (human Hb conjugated with polyethylene glycol) and Sanguinate aimed to reduce vasoconstriction. However, they faced challenges such as increased heme loss and ultimately failed in Phase 3 clinical trials [44].
- **New-generation (Encapsulated Hb):** These represent a significant leap towards biomimicry.
    - o **Hemoglobin Vesicles (HbVs):** These are cellular-structured HBOCs, encapsulating a purified and concentrated Hb solution within PEGylated phospholipid vesicles (liposomes), effectively shielding the toxic effects of molecular hemoglobin. An academia-initiated first-in-human Phase 1 clinical trial (HbV-101) commenced in Japan in 2020, assessing safety and pharmacokinetics in healthy male adult volunteers. Results indicated mild to moderate fever as a side effect [42].
    - o **ErythroMer:** This innovative product encapsulates hemoglobin within a special artificial membrane, designed to modulate oxygen affinity in a pH-responsive manner, thereby increasing its oxygen-carrying potency (reported to be 2-3 times more potent than stored RBCs). ErythroMer is also a dried red powder, offering a shelf life of at least a year, making it lighter and easier to transport for military or emergency use. Preclinical trials in rabbits have shown it to be superior to crystalloid infusion and non-inferior to stored blood. Human trials are currently being finalized [43].
- **New-generation (Naturally Polymerized Hb):** HEMO2life, derived from the extracellular hemoglobin of a marine worm, has demonstrated efficacy in organ preservation and is approved for perfusion of transplanted kidneys in the EU. Notably, no vasoactive side effects have been reported for this product, though further human trials are required to establish its safety for intravenous use [44].

**Regulatory Status:** Despite decades of intensive research and significant advancements, no HBOCs have yet received general regulatory approval from major bodies like the United States Food and Drug Administration (FDA) or the European Medicines Agency (EMA) for routine clinical use in humans, primarily due to persistent safety concerns. The progression of HBOCs from problematic first-generation products to more sophisticated encapsulated and naturally polymerized forms demonstrate a clear, iterative, and highly adaptive learning process in biomedical engineering. Initial clinical failures, driven by specific toxicities (vasoconstriction, renal damage, NO scavenging, heme loss), directly informed subsequent design modifications. The shift towards encapsulating hemoglobin or utilizing naturally large, stable hemoglobins (like marine worm Hb) is a direct attempt to mimic the protective environment of the natural red blood cell, thereby mitigating the issues associated with free hemoglobin. This indicates a deep, mechanistic understanding of the physiological challenges encountered and a targeted, biomimetic approach to product design. This continuous refinement and targeted problem-solving suggest that despite past setbacks, the field is not stagnant but is evolving with increasing sophistication. While full regulatory approval for routine transfusion remains elusive, the trajectory indicates a persistent and increasingly promising research effort, moving towards more physiologically compatible and safer designs [49,50].

## Perfluorocarbon-Based Oxygen Carriers (PFCs): Properties, Applications, and Clinical Progress

Perfluorocarbon-based oxygen carriers (PFCs) represent a distinct class of artificial blood substitutes, offering an alternative to protein-based systems. These are synthetic molecules composed solely of carbon and fluorine atoms, characterized by their remarkable capacity to physically dissolve large amounts of respiratory gases, with oxygen solubility up to 50 times that of plasma, or 50% by volume. PFCs are chemically and biologically inert, highly stable, and typically colorless and odorless. Unlike hemoglobin, which relies on chemical bonds for oxygen transport, PFCs dissolve oxygen according to Henry's law. This means the dissolved oxygen concentration at equilibrium at a given temperature is directly proportional to the gas's partial pressure. This property allows for rapid and extensive oxygen extraction and release when needed, making them efficient oxygen transporters (Table 4). As PFCs are insoluble in water, they must be formulated into kinetically stable nanoemulsions (tiny droplets with diameters typically less than 500 nm) for intravenous administration into the bloodstream.

PFCs offer several theoretical advantages, including universal compatibility (no blood typing), long shelf life, pathogen-free status, and the ability to reach hypoxic tissues effectively due to their small particle size, potentially even passing through capillaries

**Table 4:** Properties, Advantages, and Clinical Status of Perfluorocarbon-Based Oxygen Carriers (PFCs)

| PFC Type/Example | Chemical Composition | Oxygen Binding Mechanism | Key Properties | Key Advantages | Key Disadvantages/ Toxicity | Clinical Status/ Regulatory Approval |
|---|---|---|---|---|---|---|
| General PFCs | Carbon and Fluorine atoms | Physical dissolution (Henry's Law) | Chemically/ biologically inert, highly stable, high gas solubility ($O_2$ up to 50x plasma, $CO_2$ 3-4x $O_2$), insoluble in water (requires emulsion), colorless, odorless | Universal compatibility, long shelf life, pathogen-free, rapid $O_2$ release/ uptake, entirely synthetic, small particle size for tissue penetration, ultrasound contrast agent capabilities | Toxicity from surfactants, short intravascular half-life, adverse cerebrovascular effects, recruitment failures in trials, persistence in body/organ retention | No PFCOC currently approved for general clinical use |
| Fluosol DA | (Not specified) | (Not specified) | (Not specified) | (Not specified) | Secondary effects of surfactants | Discontinued |
| Oxygent | (Not specified) | (Not specified) | (Not specified) | (Not specified) | Adverse cerebrovascular effects on cardiopulmonary bypass, stroke | Discontinued (2002) |
| Oxycyte | (Not specified) | (Not specified) | (Not specified) | (Not specified) | Sponsor discontinuation due to recruitment failures | Phase 2 completed (2008), subsequent Phase 2 discontinued (2014) |
| PFOB (Perfluorooctyl bromide) | (Not specified) | (Not specified) | Well tolerated by humans 42 | Used as blood substitute, oxygen-binding capacity | (Not specified) | (Not specified) |
| PFD (Perfluorodecalin) | (Not specified) | (Not specified) | (Not specified) | (Not specified) | (Not specified) | (Not specified) |

narrower than red blood cells. Being entirely synthetic, they avoid biological sourcing issues. They also have potential as ultrasound contrast agents [38-44].

**Challenges and Clinical Status:** Early PFC products (e.g., Fluosol DA, Oxygent) faced significant issues, including toxicity related to the surfactants used in their emulsions, short intravascular half-life, and adverse cerebrovascular effects, which ultimately led to their discontinuation from clinical development. Despite their attractive characteristics, no PFCOC is currently approved for general clinical use in humans by major regulatory bodies [51].

Current research in the field focuses on optimizing nanoemulsion formulations, exploring novel stimuli-responsive behaviors (e.g., ultrasound-triggered drug release), and developing strategies for targeted drug delivery, particularly in cancer theranostics to alleviate tumor hypoxia and enhance treatment efficacy. Oxycyte, a second-generation PFC, successfully completed a Phase 2 clinical trial for traumatic brain injury in 2008. However, subsequent Phase 2 studies on its safety and efficacy, initiated in 2009, were discontinued in 2014 due to sponsor discontinuation, primarily resulting from recruitment failures. PFCs represent a fundamentally distinct approach from protein-based oxygen carriers, offering the appealing simplicity of being entirely synthetic, thereby theoretically circumventing biological complexities like immunogenicity and disease transmission. Their mechanism of oxygen dissolution is also unique and potentially advantageous for rapid oxygen delivery to tissues [38]. However, the consistent failure to achieve widespread clinical approval, despite these theoretical benefits, points to persistent challenges beyond simple oxygen transport. These issues likely stem from the complexities of formulation stability, biocompatibility of emulsifiers, and long-term biodistribution and organ retention, which can lead to undesired transient side effects. This highlights that even with a chemically inert and efficient oxygen-carrying core, the challenge of safely and effectively delivering and clearing the agent in vivo is profoundly complex. It underscores that replicating even a single function of blood safely within the human body requires meticulous engineering of the entire delivery system, not just the active component [39].

## Stem Cell-Derived Red Blood Cells: Emerging Research and Production Hurdles

The *in vitro* generation of red blood cells (RBCs) from various stem cell sources, including hematopoietic stem cells from cord blood, embryonic stem cells, and induced pluripotent stem cells (iPSCs), represents a highly promising, long-term solution to address global blood shortages and enhance transfusion safety. Stem cells offer the unique advantage of self-renewal and multipotent differentiation, allowing for continuous, potentially lifelong production of RBCs under appropriate culture conditions. This could provide an unlimited and universal source of blood, free from donor-dependent variability and transfusion-transmitted infections [48].

**Challenges:**

- **Large-scale production and cost-effectiveness:** This remains the single most significant hurdle. Current 2-dimensional cell culture systems are inefficient, yielding low cell densities (100-1000-fold less than physiological levels) and requiring high cytokine concentrations (10-1000-fold higher than physiological). The transition from current limited 2D production techniques to large-scale 3D bioreactors is crucial but requires significant technological breakthroughs. The entire process must also become at least five-fold more cost-efficient to compete with the current prices of high-quality blood products. Despite successful in vitro injection of stem cell-generated blood into a human in 2011, the practical usefulness for routine transfusion remains unproven due to these large-scale production and cost-effectiveness issues [33, 39].
- **Enucleation and Purity:** Mature RBCs are enucleated (lack a nucleus), a critical step for their function and lifespan. Achieving consistent and efficient enucleation in vitro has been a challenge, although factors like proper erythropoietin (EPO) concentration, optimal cell confluence, and appropriate culture conditions mimicking the bone marrow microenvironment are known to influence success. Current purification technologies also have limited throughput and often rely on expensive fluorescent or magnetic immunolabeling, resulting in significant cell loss (up to 70%) and quality impairment. Novel microfluidic separation techniques are being explored to sort enucleated cells from nucleated cells, aiming for higher purity (e.g., 70% purity with microfluidics, further enhanced to 99% with membrane filtration) [41, 43].
- **Immune Response and Safety:** While stem cell-derived RBCs aim to be universal and avoid immune rejection, concerns persist regarding potential irregularities from altered adult cells or spontaneous irregular growth/differentiation of embryonic stem cells. Embryonic stem cells might also trigger

an immune response or simply fail to function as expected. Controlling the growth and development of embryonic stem cells is an active area of research [45].

• **Functional Maturation:** Ensuring that *in vitro* produced RBCs fully mimic the physiological properties and functions of native human RBCs, including oxygen affinity, deformability, and lifespan, is complex. While progress has been made in differentiating stem cells into mature RBCs, achieving full functional equivalence remains an area of active investigation.

The pursuit of stem cell-derived RBCs reflects a profound understanding of the limitations of current transfusion practices and the desire for a truly regenerative and universal blood product (Table 5). The current challenges in large-scale production, cost-effectiveness, and full functional maturation are not trivial, but the continuous progress in stem cell biology and bioengineering suggests that these hurdles, while substantial, are being systematically addressed. This approach represents the most biomimetic and potentially complete solution for artificial blood, aiming to replicate the full cellular complexity rather than just oxygen transport [51].

## Clinical Trials on artificial blood (2020-2025)

The period has been marked by the initiation and progression of human clinical trials for "purple blood" (hemoglobin vesicles), predominantly in Japan.

**Japan's Lead:** Japan has emerged as a global leader in advancing artificial blood towards clinical use. Nara Medical University and Chuo University are at the forefront of these efforts.

**Early Small-Scale Studies (Pre-2022 to 2022):** Initial small-scale studies demonstrated the safety and oxygen-carrying potential of hemoglobin vesicles in human volunteers. For example, a 2022 clinical trial in Japan tested hemoglobin vesicles in a small group of healthy volunteers [39].

**Phase I Clinical Trials (Starting 2025):** A significant milestone is the planned commencement of formal Phase I clinical trials involving healthy adults by March 2025 in Japan. These trials, notably by Nara Medical University, aim to administer 100 to 400 milliliters of the artificial blood to volunteers. The primary objective of this phase is to assess the safety and tolerability of the artificial blood product. If no significant side effects are observed, the research will progress to broader studies examining efficacy [40].

**Goal for Clinical Use:** The ambitious goal in Japan is to bring this artificial blood technology to clinical use by 2030, potentially making it the first country to deploy such a product for real-world medical care [41].

**Global Landscape:** While Japan is leading in the clinical trial phase for "purple blood" (HbVs), other countries and organizations are also actively engaged in artificial blood research:

**UK Clinical Trials (2022):** In 2022, a clinical trial in the UK marked a significant step by transfusing laboratory-grown red blood cells (derived from stem cells) into human volunteers to assess their safety and longevity. This differs from the HbV approach but represents another avenue of artificial blood development [42].

**US Efforts (DARPA Funding):** The US Defense Advanced Research Projects Agency (DARPA) has been funding synthetic blood research, with human trials expected between 2028-2030 for some of these initiatives. These often focus on blood products for use in remote or battlefield scenarios [43].

**Table 5:** Comparative Overview of oxygen carriers (2020–2025)

| Technology | Composition & Delivery | Clinical Status | Shelf-Life | Key Features | Challenges |
|---|---|---|---|---|---|
| HbV (Japan, Purple) | Lipid-encapsulated expired Hb vesicles | Phase I completed; expanded trials ongoing (2025) | ~2 years at RT | Universal type, immediate oxygen carrier | Mild immune reactions, larger human trials needed |
| ErythroMer (USA) | Polymer-coated, pH-responsive Hb nanoparticles | Preclinical; human trials planned | Months | Freeze-dryable, small size, portable | Scale-up, regulatory path pending |
| Lab-grown RBCs (RESTORE) | Allogeneic stem cell–derived RBCs | Phase I transfusions underway (2022–2025) | Similar to fresh RBCs | Potential longer circulation time | Manufacturing scale, cost, long follow-up |
| PolyHeme & Recombinant Hb | Polymerized/ extracted Hb variants | Trial halted (PolyHeme), recombinant early phase | Moderate to longer | No blood type needed | Side effects (vasoconstriction), safety |

## Challenges and Future Directions

### Standardization of Protocols and Assays

A persistent and widely acknowledged challenge in KLH research is the lack of standardized immunization and immunoassay protocols across different studies. This variability is extensive, encompassing differences in KLH formulation (e.g., high molecular weight vs. sub-unit), dose, administration route, and the specific immunoassay platforms employed to assess KLH-specific responses [44]. Furthermore, there are currently no uniform sampling times or laboratory platforms established for consistently measuring the humoral or cellular KLH-specific response following immunization. This lack of uniformity extends to analytical and statistical methods, which makes it inherently difficult to generalize response sizes and compare results meaningfully across disparate studies [45].

The persistent call for standardization across KLH research highlights a critical bottleneck in translating its broad potential into consistent, comparable, and clinically reliable outcomes. The 2022 systematic review explicitly underscored the urgent need for standardized and well-controlled methodologies to induce and evaluate KLH responses. Such standardization is crucial for increasing KLH's utility as a research tool and ensuring consistency and comparability in clinical research. Encouragingly, ongoing clinical trials, such as NCT05876195 (posted in 2023), are actively addressing this issue by rigorously exploring dose-response relationships and adjuvant effects in an effort to establish more standardized protocols. Overcoming this challenge is paramount for KLH to fully realize its therapeutic and diagnostic promise, enabling its wider adoption and more reliable clinical translation [46].

### Optimizing KLH Formulations and Regimens

The diverse nature of KLH, including its two distinct isoforms (KLH1 and KLH2) and the availability of both high molecular weight (HMW) and sub-unit forms, presents significant opportunities for optimizing its application. However, this diversity also necessitates tailored formulation strategies. For instance, sub-unit KLH, while potentially offering advantages in handling or specific delivery, often requires combination with an adjuvant to achieve immunogenicity comparable to the native HMW KLH [47].

Practical challenges further complicate KLH's formulation. Its inherent propensity to aggregate and precipitate from solution can limit its ease of manipulation and reduce the efficiency of hapten conjugation. Although aggregates generally retain immunogenicity, their physical properties can be problematic for pharmaceutical development. Strategies to mitigate these issues include chemical modifications like polyethylene glycol (PEG) conjugation, which can improve solubility, or the use of alternative hemocyanins such as Concholepas concholepas hemocyanin (CCH), known as "Blue Carrier Protein," which exhibits improved solubility, especially in its activated forms [48].

The choice of conjugation chemistry also significantly impacts the efficacy and stability of KLH conjugates. As demonstrated by comparative studies, maleimide-sulfhydryl chemistry consistently yields superior results over traditional methods like glutaraldehyde, primarily due to better epitope preservation and more controlled linkage. Ongoing research, such as the 2025 study on characterizing conjugation sites, aims to further refine these processes and ensure consistent product quality. The diverse biochemical properties and varying immunogenic profiles of different KLH forms necessitate tailored formulation strategies to maximize efficacy and overcome practical challenges like solubility and aggregation. This implies that future efforts must focus on precisely engineering KLH formulations to achieve desired immune outcomes while addressing logistical complexities [45].

### Ecological Considerations

The increasing demand for Keyhole Limpet Hemocyanin in various biomedical and biotechnological applications has brought to light important ecological considerations related to its source organism, *Megathura crenulata*. As KLH cannot be synthetically reproduced and must be purified directly from the hemolymph of these marine molluscs, the rapidly growing commercial market for KLH formulations has raised concerns among fisheries biologists about the potential for overharvesting the species. Recognizing this environmental imperative, efforts are underway to ensure the sustainable sourcing of KLH. The development and utilization of sustainable maricultures of limpets, rather than relying solely on wild-harvested populations, represent a positive step towards addressing these concerns. This approach aims to balance the increasing scientific and clinical demand for this valuable biomolecule with the need for ecological preservation. The increasing demand for KLH in biomedical applications introduces an ethical and environmental imperative for sustainable

sourcing. This underscores the intersection of scientific advancement and ecological responsibility, ensuring the long-term viability of this valuable biological resource for future research and therapeutic development [49-51].

## Conclusions

Oxygen-carrying proteins display remarkable evolutionary diversity, with hemocyanin exemplifying adaptation to cold, low-oxygen environments in molluscs and arthropods through copper-based oxygen binding. Despite its biological efficiency in such niches, hemocyanin's low oxygen transport capacity, large molecular size, and strong immunogenicity render it unsuitable for human transfusion, though Keyhole Limpet Hemocyanin (KLH) is widely used as a potent vaccine carrier and cancer immunostimulant. Current artificial blood research focuses on hemoglobin-based oxygen carriers (HBOCs), perfluorocarbon-based oxygen carriers (PFCs), and stem cell-derived red blood cells. HBOCs have evolved from toxic early forms to safer encapsulated designs like hemoglobin vesicles and ErythroMer, with limited regulatory approvals but no widespread adoption. PFCs offer synthetic, pathogen-free oxygen delivery but face toxicity challenges, while stem cell-derived RBCs promise a long-term, universal supply hindered by production and maturation hurdles. The quest for viable artificial blood remains an iterative process, demanding continued innovation in biomimetic engineering and scalable manufacturing to overcome clinical and logistical barriers.

### Authors' Contribution

It is hereby acknowledged that both authors have accepted responsibility for the manuscript's content and consented to its submission. They have meticulously reviewed all results and unanimously approved the final version of the manuscript.

### Conflict of interest

The authors declare that there is no conflict of interest.

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