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Recent advances in micro-sized oxygen carriers inspired by red blood cells

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ABSTRACT

Supplementing sufficient oxygen to cells is always challenging in biomedical engineering fields such as tissue engineering. Originating from the concept of a 'blood substitute', nano-sized artificial oxygen carriers (AOCs) have been studied for a long time for the optimization of the oxygen supplementation and improvement of hypoxia environments *in vitro* and *in vivo*. When circulating in our bodies, micro-sized human red blood cells (hRBCs) feature a high oxygen capacity, a unique biconcave shape, biomechanical and rheological properties, and low frictional surfaces, making them efficient natural oxygen carriers. Inspired by hRBCs, recent studies have focused on evolving different AOCs into microparticles more feasibly able to achieve desired architectures and morphologies and to obtain the corresponding advantages. Recent micro-sized AOCs have been developed into additional categories based on their principal oxygen-carrying or oxygen-releasing materials. Various biomaterials such as lipids, proteins, and polymers have also been used to prepare oxygen carriers owing to their rapid oxygen transfer, high oxygen capacity, excellent colloidal stability, biocompatibility, suitable biodegradability, and long storage. In this review, we concentrated on the fabrication techniques, applied biomaterials, and design considerations of micro-sized AOCs to illustrate the advances in their performances. We also compared certain recent micro-sized AOCs with hRBCs where applicable and appropriate. Furthermore, we discussed existing and potential applications of different types of micro-sized AOCs.
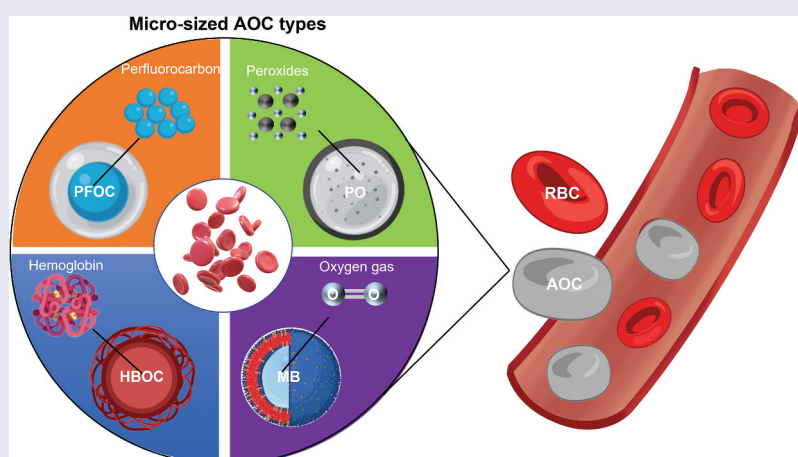
1. Introduction

Oxygen is a necessary substance for aerobic cell survival and proliferation and an important factor in numerous metabolism processes and pathologies in the body [1]. Owing to the limited oxygen solubility in the blood serums, culture media, and scaffold materials in tissue engineering, providing an efficient and precise oxygen supply remains a constant challenge in various biomedical fields [2] such as oxygenation

therapy and cancer therapy [3], as well as in blood substitutes for transfusion.

Artificial oxygen carriers (AOCs) have long been studied as an approach to optimizing oxygen supplementation and improving hypoxia environments *in vitro and in vivo* [4]. In fact, the concept of AOCs was originally derived from red blood cells (RBCs), i.e. the natural oxygen-delivering cells in whole blood. AOCs were originally developed as blood substitutes

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to compensate for the drawbacks of allogeneic blood transfusion, including shortages in emergency situations such as disasters and wars, limited numbers of healthy donors, complicated processing, short shelf lives [5,6], adverse effects such as immunosuppression and infectious disease transmission [7], and religious and ethical concerns [8].

Aiming to utilize the high oxygen binding and affinity of hemoglobin (Hb) in RBCs, purified stroma-free and endotoxin-free Hb from bovine or human sources became the basis material for the earliest AOCs in the 1950s [9]. In the early forms of Hb-based oxygen carriers (HBOCs), the Hb was cell-free, i.e. chemically modified or crossed-linked with a molecular weight under 500 Da [10]. The Cell-free Hb was administered to several US navy patients in the 1950s, but cardiovascular complications were noted as a side effect [11]. In 1957, multiple materials were used to encapsulate Hb, such as nylon, collodion, gelatin, and silicone. The first Hb-loaded liposome was reported in 1977; the Hb was loaded into the phospholipids, fatty acids, and cholesterol liposomes [12,13].

Subsequently, chemically surface-modified (polymerized or conjugated) Hb was developed as HemAssist in 1985. Polyheme was clinically developed in 1996 as a polymerized version of Hb free of tetramer Hb [7]. However, the clinical uses of early HBOCs were accompanied by many side effects including vasoconstriction, methemoglobin generation, and acute anemia [14]. In view of these challenges, novel nano-seized Hb-based AOCs are now being energetically studied [15,16].

The other earliest type of AOCs were the perfluorocarbon (PFC)-based oxygen carriers (PFOCs) introduced by Clark and Gollan in the 1960s [17]. PFCs are a group of synthetic chemicals. They both hydrophobic and lipophobic owing to fluorine's low polarizability. Owing to the low van der Waals interactions between PFC molecules, they can dissolve a large quantity of oxygen [18]. In addition, PFCs transport oxygen molecules according to Henry's law, in which the loading and release of oxygen are controlled by the partial pressure of oxygen in the environment. The outstanding physical and chemical properties of PFCs make them effective materials for AOCs [19]. In hypoxic tissues, when both PFCs and RBCs are simultaneously present in the circulation system, the PFCs release oxygen to guard the Hb-bound oxygen until it reaches the hypoxic tissues. Owing to the poor solubility of PFCs in water, Fluosol-DA (Green Cross Corp., Japan) was the first PFC nano-emulsion system to receive FDA approval in 1989. It included 20 wt.% (10.6% volume) of perfluorotripropylamine along with Pluronic F−68, egg yolk phospholipid, and potassium oleate. As a result of its short shelf life, pulmonary complications, reduced platelet, increased white blood cell (WBC) counts, and decreased neutrophil counts, it was discontinued in 1994 [20]. The 2nd generation of PFOCs (such as Oxygent®) eventually met the same fates as their precedents [7]. Although PFOCs have a lower solubility of oxygen than that of HBOCs under the same partial pressure, they have the advantages of being pasteurized more easily and being chemically inert to oxygen; as such, they can withstand more physical, metabolic, and chemical stimuli and can be preserved for a longer time. However, the polydispersity of previous PFOCs and toxicities of the poloxamer or phospholipids surfactants have been shown to activate the complementary system and macrophage activity, leading severe adverse reactions such as influenza-like symptoms [21]. Moreover, the nano-sized PFC emulsions were rapidly cleared from circulation by the reticuloendothelial system, leading to short circulation times (half-life 6 – 12 hours) [22]. Unfortunately, all of the previous HBOCs and PFCs nano-emulsion products have discontinued their manufacture or been disapproved by FDA; no reliable AOCs for replacing human blood exist in the market today. Correspondingly, greater efforts are focused on both HBOCs and PFOCs nowadays [23–25].

In addition to nano-sized AOCs, micro-sized AOCs have emerged and gained profound attention. As their sizes usually range from a few to hundreds of microns, micro-sized AOCs can be distinguished from their nano counterparts in cultured tissues without internalization by cells or penetration into the extracellular matrix [26]. Micro-sized AOCs can also help overcome some of the inherent limitations of nano-emulsion HBOCs and PFOCs, such as the toxicity of the surfactants and short circulation period.

Recently, several types of micro-sized AOCs have been proposed, such as microspheres, micro-sized emulsions, and microcapsules with core-shell architectures. Owing to the protective outer shell, the microcapsule structure possesses general merits such as protection of compounds in the core, controlled release of the core material, and an enhanced shelf life [27]. The shell can act as a barrier between the oxygen-carrying core material and surroundings of the AOCs. Thus, we expect that high-oxygen-permeable materials will be desirable for the shell, as its thickness must be strictly controlled. In addition to providing oxygen permeability for rapid oxygen absorption and desorption, the shell can also be readily engineered in terms of its morphology, porosity, and biomechanical properties. Surface coatings or modifications (such as the conjugation of a receptor on the shell for specific site-targeting) is also possible [28].

Another unique aspect of micro-sized AOCs is their potential for mimicking the morphology and rheology of RBCs. RBCs can circulate smoothly via blood capillaries and vessels owing to their

extraordinary deformability and shear-thinning properties. RBC-mimicking AOCs should therefore have excellent biomechanical properties, such as in the deformability and viscoelasticity of their suspension. This will help them provide longer intravascular circulation based on the lower cellular adhesion and sufficient oxygen supply to peripheral tissues based on the better biodistribution. Then, they will have the potential to provide oxygen delivery functions similar to those of human RBCs, demonstrating, e.g. minimal accumulation in organs, non-aggregation, and sustainable oxygen supplementation. These can be particularly useful in many biomedical fields, such as perfusion culturing *in vitro* and transfusion alternatives *in vivo*.

In general, the fundamental biomaterials of micro-sized AOCs are chemicals for stabilizing or producing oxygen. The general approach is mainly based on three major oxygen-binding or oxygen-generating biomaterials: Hb, PFCs, and peroxides (POs). Their products can be categorized into four mainstream AOC types: HBOCs, PFOCs, POs, and oxygen microbubbles (OMBs). Even though those AOC types share some common techniques regarding the fabrication and oxygen delivery functions, the microparticles in each AOC category also have uniqueness in terms of their biomaterial properties, oxygen-releasing mechanisms, and limitations, making them suitable for certain biomedical applications. For instance, POs are frequently embedded in artificial scaffolds for tissue regeneration, whereas OMBs are usually injected into a targeted site for short period of oxygen therapy. Figure 2 shows the general formulation and oxygen-delivering strategy for each micro-sized AOC type inspired by RBCs.

In this review, we deliberately discuss the recent advances in micro-sized AOCs by summarizing the most illustrative examples of oxygen-delivering microparticles of different types from the past decade. The state-of-the-art fabrication methods, material compositions, and intended applications are evaluated and elaborated upon. Then, we provide design criteria and perspectives for the development of the next generation of micro-sized oxygen carriers.

2. Natural oxygen carriers – Red blood cells (RBCs)

Whole blood works as the medium for transporting nutrients and oxygen throughout the human body. It mainly contains a plasma medium, WBCs, RBCs, and platelets [29]. RBCs comprise 40% of the blood and serve as the major oxygen-carrying vehicles. They are naturally engineered to circulate from the arterial vessels (up to ~100 μm) to the peripheral capillary vessels (down to ~5 μm) for approximately 120 days [30]. A healthy RBC has a unique interchangeable morphology and core-shell (Hb-cell membrane) structure as shown in Figure 1, providing it with an excellent deformability and oxygen capacity to meet the oxygen demands of each tissue and cell. Those properties are utilized by RBCs as efficient cell vehicles. Therefore, comprehension of the mechanisms and constitution of RBCs can help in the design and optimization of micro-sized AOCs.

2.1 Size and shape

As a microcapsule with a fluidic core and elastic membrane, the RBC's shape and size is closely related to its biomechanical and physical properties, as well as the forces subjected to it from the surroundings (such as shear stresses). A healthy human RBC subjected to zero external forces has the shape of a biconcave discoidal with a size of approximately 7.5 μm in diameter and 2 μm in thickness [31]. An RBC has multiple morphologies in its relaxed state, including anisocytosis (variation in size) and poikilocytosis (variation in shape). Under various pathological conditions, some of the abnormal shapes dominate and are often associated with different
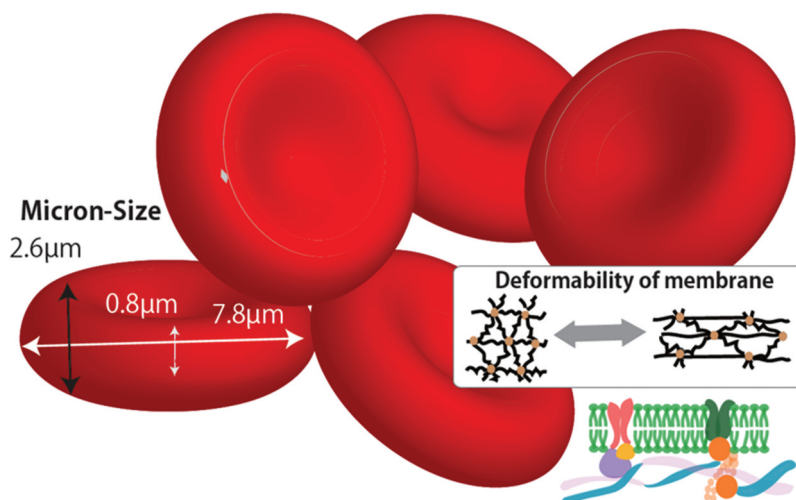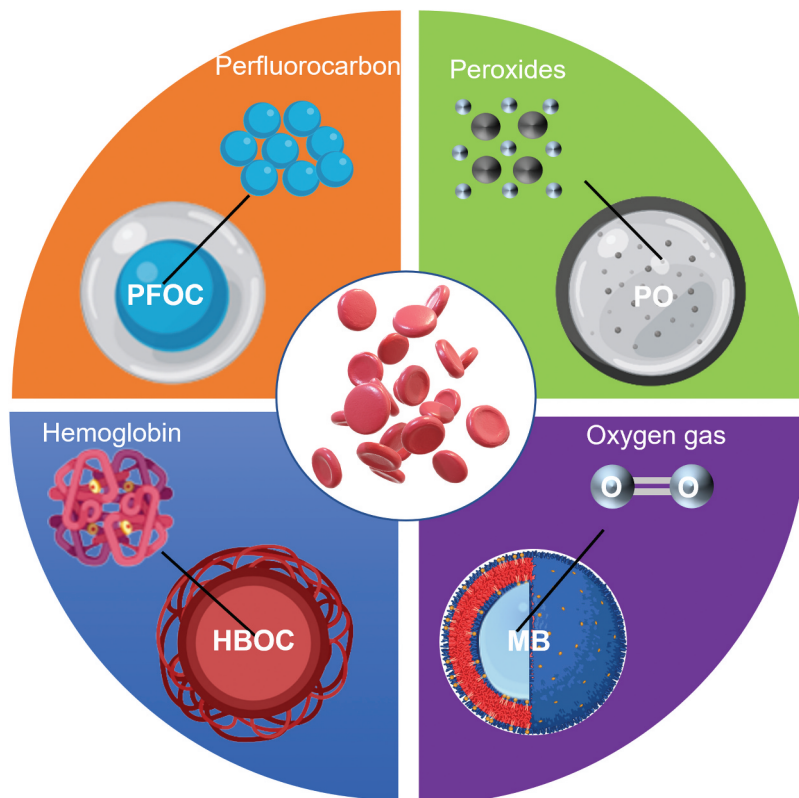

Figure 1. Human red blood cells (hRbcs).

Figure 2. Micro-sized AOCs inspired by hRbcs.

diseases [32]. For example, Figure 3 shows typical shapes observed in the blood smear examinations of patients with COVID−19 [33]. As another example, a decrease in the fraction of RBCs with biconcave shapes often leads to several diseases such as sickle cell anemia, which has still no effective treatments in most cases. Numerous studies have considered the conditions and indications of different RBC morphologies, but healthy RBCs can deliver oxygen the most efficiently, owing to the high deformability and high surface area of the gas exchange as they circulate through the body. Such high deformability helps the healthy RBCs morph into different shapes according to their flow conditions, leading to optimal blood rheological properties such as shear-thinning (which helps them pass through capillaries without occlusion).
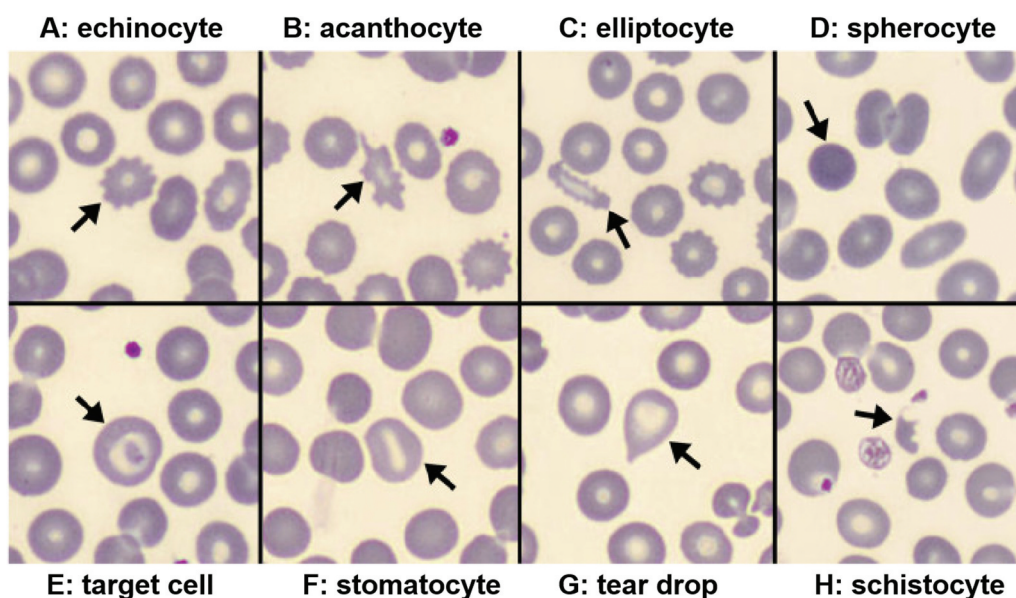
Figure 3. Typical abnormal RBC morphologies observed in the peripheral blood samples from COVID − 19 patients, adapted with permission from [33]. Copyright 2022 Marchi, Bozzini, Bertolone, Dima, Busti, Castagna, Stranieri, Fratta Pasini, Friso, Lippi, Girelli and Vianello.

In view of the lessons learned from the sizes and shapes of human RBCs (hRBCs), the morphology consistency and size uniformity of the particles may become important to the design of micro-sized AOCs for clinical use.

2.2. Microcirculation

The human body contains a complex capillary network where blood flows constantly while carrying its vehicle components. Blood, as a non-Newtonian fluid, can flow steadily through complex microvascular structures owing to its shear-thinning behaviors [34]. The viscosity of the blood is dependent on various components such as hematocrit and plasma, as well as the viscoelastic properties of formed elements [35]. As RBCs comprise the most abundant formed elements in the blood, the viscosity of blood increases almost linearly with the hematocrit [36]. Even in the narrowest blood capillaries of 3–4 μm, RBCs can still pass without impediment owing to the deformability given by the cell membrane as supported by the spectrin network on their surface (as mentioned in the earlier section). Owing to this deformability, RBCs are not constricted in their shape as they face high shear stresses and intracapillary resistance; they deform themselves into different shapes (including parachutes and slippers) at different velocities [37].

Notably, however, obstructions can occur during RBC circulation through the microvasculature when the integrity or morphology of the RBCs is affected by hemolysis, inflammation, adhesion, and damage. As one of the most famous examples, sickle cell disease alters the rheology properties of blood by impairing the RBCs' ability to deformation. As a result, the viscosity of the blood increases, leading to a loss of microvascular function [38]. Thus, the physical and mechanical properties of RBCs are tightly related to their behaviors in microcirculation. The rigidity of RBCs has been proven to affect their intracapillary transport resistance, pulmonary arterial pressure, and oxygenation [39–41]. In general, the balance of the oxygen capacity and stiffness of RBCs are naturally optimized so that they can continuously release oxygen during microcirculation. The microcirculation phenomenon has been extensively studied; in this context, microfluidics have been demonstrated as an effective approach to accurately simulating the microcirculation of RBCs [42]. RBC capillary models would also be useful in the design of the parameters for micro-sized AOCs (such as their shape and softness) to obtain the desired rheological properties for circulation in capillary-size vessels.

2.3 Storage as packed red blood cells (PRBCs)

Traditionally, whole blood is processed and stored in blood banks for future transfusion after collection from donors. To eliminate febrile and allergic reactions and improve the storage life, the general prepossessing includes anticoagulation treatment, pathogen reduction, WBC reduction (leukoreduction), and other specialized storage procedures [43–45]. Nevertheless, the expiration of unseparated whole blood remains relatively fast, i.e. in the range of 20–30 days when stored at 2–6°C in a refrigerator (depending on the anticoagulant type) [46].

Alternatively, the *ex vivo* storage of whole blood can be replaced using packed RBCs (PRBC), where 70% of the plasma is removed by centrifuge. The storage life of an PRBC can be prolonged to 42 days at 1–6°C [47]. PRBCs are highly concentrated RBCs without plasma; thus, the probability of a transfusion-associated circulatory overload is lower than that with a whole blood transfusion [48]. Leucodepletion of PRBCs is also recommended before transfusion to prevent primary alloimmunization to human leukocyte antigens; however, this significantly increases the cost [49]. In addition, different anticoagulant or processing techniques during the separation and processing of RBCs from the whole blood may have various adverse effects on RBC oxygen-carrying functionality (e.g. affecting morphology changes and hemolysis) [50,51].

Furthermore, many dysfunctional elements can accumulate during the storage of PRBCs, including red blood cell-derived microparticles, aggregated Hb, band 3, and lipid raft proteins, which are biomarkers for many types of diseases [52–54]. The causes of RBC degradation are progressive with the hypothermic storage duration and are often collectively referred to as RBC storage lesions, leading to increasing Hb denaturation and various physiological impacts on the transfusion recipients [55]. Moreover, recent studies have shown that using older PRBCs can increase the coagulation potential and lead to hemorrhagic shock [47]. After all, donor-related variables inevitably induce variability in the RBC component quality and play a role in the uncertainty of the blood storage life [56]. All of the evidence from the related laboratory and clinical studies suggests that greater efforts needed to be spent on developing more advanced technologies for collecting, processing, and storing either whole blood or PRBCs to make transfusion safer and more cost-effective. The complex handling and storage of PRBCs and whole blood altogether have contributed to the high prices of blood transfusion for patients. Though the procedures and resources in each country and facility are different, in United States hospitals, the average cost of blood transfusion increased from an already high estimation of $155 per unit in 1990s [57] to approximately $300 per unit in recent years [58].

In comparison to PRBCs, AOCs (and especially AOCs with core-shell structures) have shown much longer shelf lives depending on the type owing to the simplicity of the composition, stability, and steriliz-ability, contributing to their biochemically inert shells. These facts emphasize the value of micro-sized AOCs as potential RBC alternatives in the future.

3. Fabrication methods for micro-sized artificial oxygen carriers (AOCs)

The advances in micro-sized AOCs also reflect the maturity and development of novel fabrication meth-ods for achieving the desired morphology with mono-dispersity. The major morphologies of micro-sized AOCs are microfibers, microparticles, and microcap-sules. No standards exists for selecting techniques for different types of AOCs. In general, each method has advantages and limitations. In this section, we focus on recent advances in the technical aspects and unsolved issues in preparing micro-sized AOCs. Understanding the development of preparation meth-ods based on past studies can help researchers decide the appropriate fabrication technologies for the next generation of micro-sized AOCs.
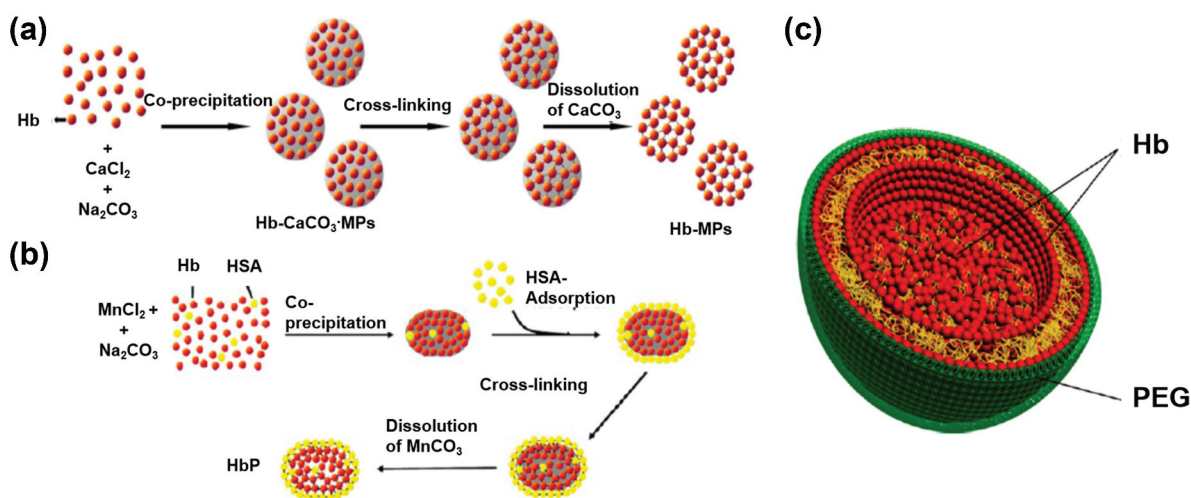
3.1 Coprecipitation and chemically crosslinking of hemoglobin (Hb)

Micro-sized HBOCs are often developed by chemical crosslinking. Hb was first precipitated by salts and micro-sized Hb particles were fabricated by subsequent chemical crosslinking, as shown in Figure 4(a). The salts were removed by dissolution and glutaraldehyde was commonly used for the crosslinking [59]. In another study, albumin was determined as biocompatible and

suitable for dispersing particles; it was used for copre-cipitation with Hb, as shown in Figure 4(b) [61]. Kai et al. [62] encapsulated Hb within a crosslinked bovine serum albumin (BSA) shell, where the cross-linkage of the albumin proteins was induced by genipin as pre-viously described by Butler et al. [60]. Li Li D et al. coated the surfaces of 4 μm-sized AOCs prepared by the coprecipitation method with polyethylene glycol (PEG) [63], as shown in Figure 4(c).

3.2 Electrospinning/Electrospraying

Electrospinning and electrospraying are electrohydro-dynamic processes in which single or multiple electri-cally charged polymer solution jets are sprayed or spun for the formation of nano-sized to micro-sized fibers and particles, respectively [64]. Usually, the particles or fibers form and solidify and are collected in an ultrasonic aqueous bath before solvent evapora-tion. This technique has been extensively used in the last decade to prepare AOCs. Single axial electrospin-ning or electrospraying is used to generate microfibers or microparticles with single phases. For instance, Erlane et al. [65] produced a polycaprolactone (PCL) microparticle loaded with nano-sized calcium PO (CPO) oxygen-generating microparticles by electro-spraying at a positive voltage of 12 kV. Those micro-particles ranged in size from 5 to 15 μm and were later encapsulated together with cells in the hydrogel for enhanced cell expansion. Similarly, Sajedeh et al. [66] fabricated polylactic-co-glycolic acid (PLGA) micro-particles loaded with nano-CPO with average dia-meter of 5.3 μm by electrospraying at a constant voltage of 15 kV. Notably, the electrosprayed PLGA/ CPO microparticles presented a biconcave disk-like morphology in spite of a wide size distribution.

Figure 4. Fabrication of AOCs by coprecipitation and subsequent chemical cross-linking. (a) Fabrication scheme of hemoglobin microparticles, reprinted with permission from [58]. Copyright 2012, American Chemical Society (b) Fabrication scheme of Hb particles, reprinted with permission from [59]. Copyright 2013, American Chemical Society (c) Schematic representation of the assembled Hb microspheres with the surface modified by PEG, reprinted with permission from [60]. Copyright 2012, American Chemical Society.

Morais et al. [67] prepared mono-dispersed PCL/CPO microparticles with diameters of $17.0 \pm 0.3\,\mu m$ at a positive voltage of 12kV.

Coaxial electrospinning and electrospraying represent novel strategies for fabricating core-shell micro-sized AOCs. In addition to a single nozzle, Zhang et al. [68] also employed a coaxial nozzle to prepare both single- and double-walled PCL/CPO microparticles with diameters of $26.4 \pm 5.1\,\mu m$ using coaxial electrospraying. Compared with the electrosprayed single-walled PCL/CPO microparticles, the double-walled structure provided an additional barrier to the outside water and slowed down the release of oxygen. Recently, Ma et al. [69] fabricated a perfluorotributylamine (PFTBA)-PCL core-shell microfiber system using coaxial electrospinning with concentric spinnerets with an outer needle diameter of 1.2 mm and inner needle diameter of 0.3 mm to prolong the oxygen release of the PFTBA for nerve regeneration. A maximum positive voltage of 16 kV steadily generated core-shell microfibers with diameters of $7.5 \pm 2.2\,\mu m$ and $3.2 \pm 2.4\,\mu m$.

Although electrospraying and electrospinning have the advantages of a high encapsulation rate and high production rate, one typical issue is the difficulty in controlling the size. In recent examples of produced micro-sized AOCs, the experimental conditions were individually optimized without a standard guideline. Several parameters can be optimized in the processes of electrospinning or electrospraying, such as the voltage, flow rate, and height. Among those parameters, a high voltage ranging from 5 kV to 20 kV has often been required to drive a polymer solution ejected at a constant speed; this has correspondingly been limited by machine strength and safety concerns. Therefore, future developments in electrodynamic processing in terms of the fundamental equipment such as the quality of micronozzle array [70] are likely to improve the overall production quality of micro-sized AOCs. Moreover, coaxial electrodynamic processes are interesting because they can be arranged to produce microparticles with multiple layers [71]; this can help in the design of novel micro-sized AOCs with multi-layered structures with potentially advanced properties.

### 3.3 Mechanical emulsification

Emulsification is one of the simplest and most widely used methods for the preparation of AOC emulsions from nano- to micro-sizes. It was used as early as in the 1st generations of HBOCs and PFOCs [8]. To produce AOCs, an oil-in-water single emulsion and water/oil/water double emulsion are usually formed to produce the microparticles and microcapsules, respectively. When preparing AOCs composed of polymeric biomaterials (such as polymer-shelled microcapsules), solvent evaporation is often applied after the emulsification

process [72]. The interfacial tension between the phases plays a critical role in the size distribution, dynamic formation, and encapsulation rate of the emulsion droplets. Surfactants consequently become an important factor, because they can lower the interfacial tension between the oil phase and water phases, as well as prevent the coalescence of the microdroplets [73]. Recently, Fu et al. [74] studied the effects of the Pluronic surfactant type and concentration on preparing micro-sized PFC emulsions. They consequently determined the appropriate surfactant for stabilizing a micro-sized PFC emulsion with narrowest size distribution and highest stability. Zarzar et al. [75] precisely controlled the droplet morphology in a perfluorocarbon-in-hydrocarbon-in-water or hydrocarbon-in-perfluorocarbon-in-water complex emulsion system from a Janus shape to a core-shell by changing the surfactant concentration, type, and temperature. Thus, they suggested the possibility of developing PFOC microcapsules with customized shapes by emulsification in the future. Another common emulsification parameter especially important in double emulsions is the phase composition or concentration ratio of each species. Nejati et al. [76] studied the influences of process parameters on micro-sized poly (l-lactic acid) (PLLA) loaded with a polyvinylpyrrolidone/hydrogen PO (PVP/H_2O_2) mixture. They tested the effects of different concentrations of PLLA, molar ratios of H_2O_2 to PVP, and phase volume ratios (from aqueous to organic) on the size, encapsulation rate, loading amount, and initial burst release rate. The results helped them to design a multi-optimization strategy for fabricating the desired microcapsules by emulsification.

Nevertheless, remaining surfactant molecules on the particle surface can occasionally cause side effects and aggregations of particles; thus, minimizing the use of surfactant has becomes a challenging study topic in the manufacturing of AOCs.

3.4 Shirasu-porous-glass (SPG) membrane emulsification

Membrane emulsification is another effective strategy for controlling the sizes of microparticles or microcapsules by applying a constant pressure to force dispersed permeation through the membrane pores to the continuous phase [77]. In particular, Shirasu-porous-glass (SPG) membrane emulsification is frequently used to produce microcapsules or microspheres with precise size control in the areas of food science and AOCs. Besides its high encapsulation rate and narrow size distribution, SPG membrane emulsification also has the advantages of high productivity, low energy costs, and a wide span of pore sizes [78]. In recent years, Lai et al. [79,80], Fu et al. [74,81], and Hashimoto and Ohta et al. [82] successfully prepared mono-dispersed micro-
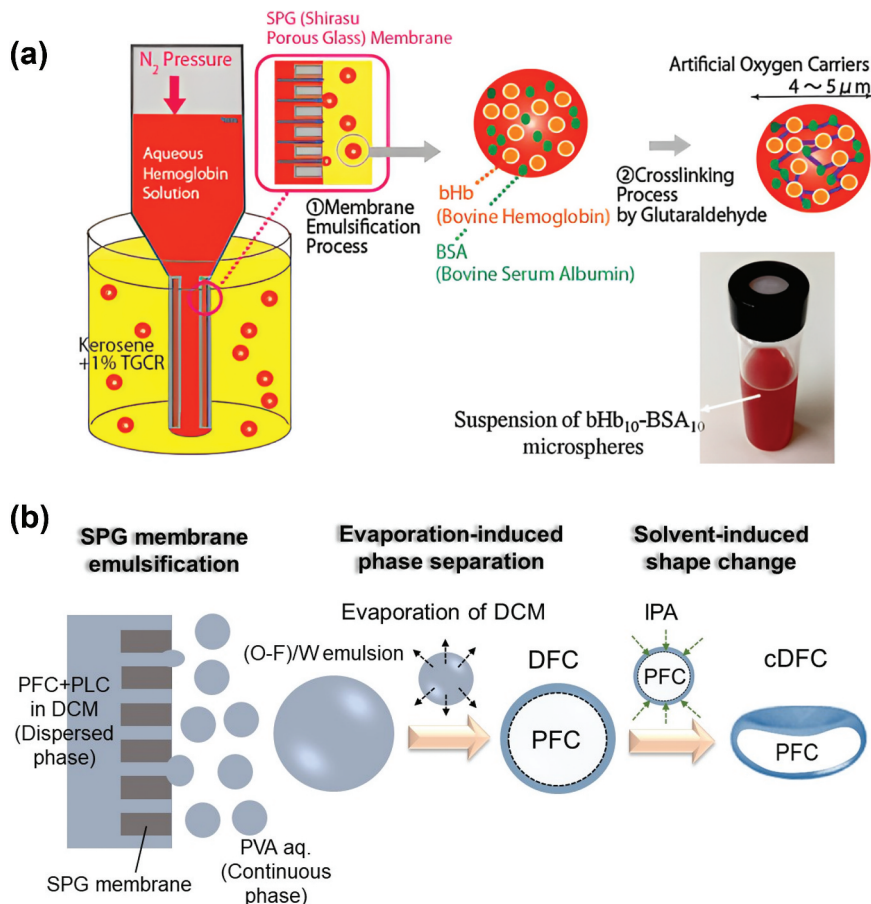
Figure 5. Preparation of AOCs by SPG membrane emulsification. (a) Hb/BSA microspheres reprinted with permission from [79]. Copyright 2015 Elsevier B.V. (b) PLC shell/PFOB core microparticles reprinted with permission from [73]. Copyright 2019 American Chemical Society.

sized PFOCs and HBOCs with sizes under 10 μm using SPG membrane emulsification. Although it has been repeatedly reported that the size of the microparticle is linearly correlated to the membrane pore size between 1μ m and 20 μm, the actual dependency varies according to the membrane type, material, and solvent system [83,84]. After the fabrication of core-shell particles, isopropanol (IPA) can be used to induce shape changes to fabricate concaved deformable PFC-based oxygen carriers with similar sizes as RBCs and a narrow size distribution, as shown in Figure 5(b). The concave-shaped PFOCs have a lower elastic modulus and higher flexibility than the original microparticles. Although the shape change has been hypothesized as being owing to partial fluidization owing to the sorption of IPA by the poly(lactic acid-co-ε-caprolactone) (PLC) polymer shell, further research into the actual mechanisms of the shape change is expected.

3.5 Flow focusing

Flow focusing has typically been used in the settings of microfluidic or T-junction devices to produce micro-droplets based on Plateau – Rayleigh instability, with the advantage of a highly uniform size [85]. The production of OMBs by flow- focusing techniques has also been applied as post-processing after a sonication preparation of microbubbles to control the size range of and shell thickness of the synthesized OMBs [86]. Elena et al. [87] showed success in generating mono-dispersed, non-coated, air-filled microbubbles by simple flow-focusing via a modified microfluidic device. However, the microbubbles were not stabilized by surfactants or phospholipids for practical uses as AOCs.

Until very recently, only a few studies had applied flow focusing techniques to microfluidic chips to manufacture OMBs specifically for oxygen delivery purposes. Ashwin et al. [88] used a microfluidic device to produce microbubbles coated with different membranes on demand and in real time. Their device incorporated flow focusing geometry and could control the hemodynamic parameters, such as the blood saturation and blood oxygen tension in real-time by instantly adjusting the infused oxygen concentration.

A common drawback of the microfluidic flow focusing technique is the high cost of the dedicated microfluidic chips used as focusing chambers. Coaxial flow focusing (CFF) is an emerging technique which can be applied in the preparation of core-shell micro-sized AOCs. In this technique, two phases (liquid-liquid or

liquid-gas) from feed tubes meet and pinch-off at the capillary exit of a concentric needle. Recently, Ma et al. [89] applied a novel gas-driven CFF process to obtain PFC-core, lipid-shelled, and indocyanine green (ICG)-loaded microcapsules with a diameter of 37.5 ± 4.3 μm. The coaxial needle assembly was manufactured by laser welding technology including an inner needle of inner diameter = 0.4 mm and an outer needle of inner diameter = 1.0 mm. When the outer liquid solution (lipid suspension) and inner liquid solution (IGC dissolved PFC solution) were forced by a constant pressure of 1.5 kPa, a stable cone-jet was formed after passing the small orifice of diameter = 0.6 mm and spontaneously broke into the microcapsules with nearly 100% encapsulation and narrow size distribution. Meanwhile, Xu et al. [90] applied a similar process to produce micro-sized core-shell PFOCs with lipid shell and LLL12 loaded PFC-core, in the size range of 4.9 ± 1.5 μm. In this study, they discovered the relationship between average droplet size and inner phase flow rate, outer phase flow rate, and focusing phase pressure difference, which was correlated to Laplace pressure.

One year later, Liu et al. [91] used a liquid-driven CFF process to form mono-dispersed ethoxylated trimethylolpropane triacrylate (ETPTA)-shell, Hb-core microdroplets of with a diameter of 32.8 ± 1.8 μm. Inside the pressure chamber, a focusing aqueous phase containing the surfactant poly(vinyl alcohol) (PVA) drove the inner liquid phase (purified Hb in phosphate-buffered saline) and outer oil phase (ETPTA in ethanol) to produce the core-shell micro-sized HBOCs.

Both single and coaxial CFF techniques share the advantage of a narrow size distribution, whereas the latter usually has a lower cost and is suitable for producing core-shell micro-sized AOCs. If the diameter of the products can be controlled by customizing the parameters of the needles and flows, CFF techniques will be useful for producing RBC-mimicking AOCs with core-shell structures in the future.

3.6 Layer-by-layer method using template particles

To fabricate RBC-mimicking oxygen carriers or RBC-mimicking hollow templates potentially capable of loading oxygen, the main technical challenge concerns the fabrication of concave or biconcave microcapsules with moderate size distributions and desired biomechanical properties. This usually requires more sophisticated preparation techniques or post-possessing of the microparticles. The research trends regarding RBC-mimicking templates helped advance those for RBC-mimicking oxygen carriers in the past decade as tools for studying blood particulate analogue fluids [92]. For example, techniques used to fabricate RBC-mimicking templates also succeeded later in preparing RBC-mimicking oxygen carriers.

'Layer-by-layer' (LbL) technology utilizes the attraction of the opposite surface charges between the template core and layer and between each layer to deposit one or multiple layers on a template core [93]. Then, the template core coated by the layers can be removed by dissolution, resulting in hollow microcapsules [94]. The shape of the template core largely determines the morphology of the final product. Nishit et al. [95] used electrospinning and an LbL treatment to prepare RBC-shaped HBOC particles with a diameter of 7.2 μm from their original microspherical morphology, as illustrated in Figure 6. After synthesis of mono-dispersed PLGA microspheres by electrospinning, the PLGA templates were incubated in 2-propoanl to induce shape changes. After a subsequent LBL coating of nine layers of Hb/BSA and poly(allylamine hydrochloride)/BSA (PAH/BSA) on the PLGA discoidal template, the resultant microcapsules were incubated in
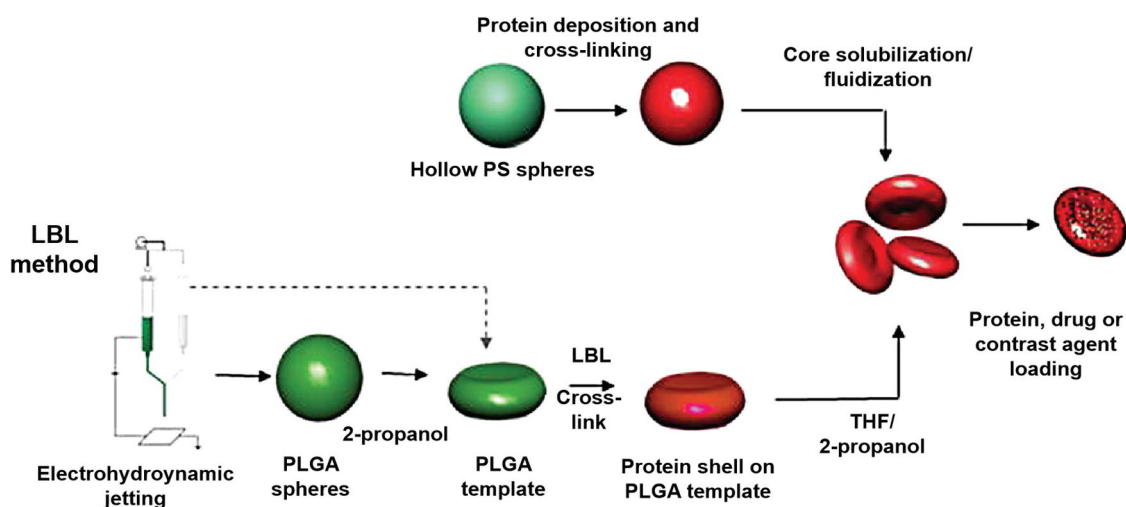

Figure 6. Fabrication process of RBC-mimicking micro-sized AOCs from PS template by LbL method, adapted with permission from [94]. Copyright 2009 National Academy of Science.

a cosolvent of tetrahydrofuran and 2-propoanl to remove the PLGA core for the formation of hollow microcarriers. These microcarriers had an elasticity of 93 ± 42 kPa, similar to that of RBCs. Shaillender et al. [96] applied the LBL technique to produce a RBC-mimicking carrying system by taking erythrocyte ghosts as templates and coating them with biocompatible poly-electrolyte layers. Their products maintained the biconcave shape of the RBCs to a great extent with a highly uniform diameter of 6.7 μm and thickness of 2.8 μm. The pre-loading of oxygen-generating or oxygen-releasing agents to the erythrocyte ghost templates could potentially make these microcapsules micro-sized AOCs. Recently, Guo et al. [97] synthesized rebuilt red blood cells (RRBCs) with modular designs using more complicated LbL approach with a similar RBC ghost as the template. Owing to the modular constructure of the RRBCs, Hb could be easily loaded into the RRBCs for oxygen delivery.

Instead of using RBC derivatives as a template, She et al. [98] prepared discoidal shaped multilayer micro-capsules with a diameter of 6.7 ± 0.5 μm and thickness of 2.8 ± 0.5 μm using a covalent LbL assembly of deprotonated PAH and glutaraldehyde onto a discoidal $Ca(OH)_2$ template. Dextran sulfate was added to change the prismatic $Ca(OH)_2$ particles into a biconcave discoidal shape as the desired template and then were dissolved by HCL after the assembly of 10 layers of PAH. Yu et al. [99] utilized the same dextran-induced shape change on an inorganic $Ca(OH)_2$ template to fabricate biconcave RBC-sized HBOCs by coprecipitation of Hb on the $Ca(OH)_2$.

Solvent-induced shape changes have been used in effective strategies for processing microparticles or microcapsules into concave shapes, and to fabricate polymer or inorganic templates into discoidal shapes (as in the above-mentioned studies). For instance, 2-proponal was hypothesized to induce the collapse of PLGA microspheres by the partial solubilization and fluidization of the core [95]. In spite of the simple immersion or incubation procedure, the mechanisms of the shape changes have yet not been fully interpreted, and the choice of solvent remains highly specific to the template material.

3.7 Particle replication in non-wetting templates (PRINT)

The 'Particle Replication In Non-Wetting Templates' (PRINT) technique is an imprint-based lithographic technique which utilizes elastomeric, low-surface-energy fluoropolymer molds on a high-surface-energy sheet, allowing for the molding of monodisperse and shape-specific particles [100]. This technique is promising for fabricating the desired shapes of microcapsules owing to its highly customized mold. Merkel et al. [101] first used PRINT to fabricate hydroxyethyl

acrylate hydrogel microparticles to mimic the shape and elastic properties of RBCs. By adding different amounts of cross-linker to the hydrogel solution, they adjusted the rigidity of the resultant hydrogel particles from 63.9 to 7.8 kPa, similar to the rigidity of RBCs. Owing to a meniscus formed during the filling of the polymer solution in a mold with size of 2–3 μm, the hydrogel particles swelled to discoidal shapes after hydration with diameters of 5.2 to 5.9 μm and thicknesses of 1.2 to 1.5 μm (similar to those of RBCs). Chen at al [102]. extended this method to fabricate RBC-mimicking HBOCs by incorporating bovine Hb into a PRINT-prepared RBC-mimicking microgel. They utilized a similar procedure to prepare triethylene glycol acrylate (TEGA) and 2-carboxylethyl acrylate crosslinked hydrogel particles by using patterned wells with a diameter of 2 μm and thickness of 0.6 μm. After the swelling and transfer to a PBS solution, the hydrogel hydrated to approximately 6.3 μm in diameter and 1.8 μm in thickness, with a narrow size distribution. Although PRINT techniques can produce nanoparticles or microparticles with consistent sizes and shapes for encapsulating small-molecule drugs, RNA, and AOCs, the yield is directly limited by the number of mold cavities.

4. Innovative designs and functions of recent micro-sized AOCs

Based on the understanding of RBC mechanisms, certain micro-sized AOCs have shown improved performance in different aspects in recent years. Various parameters can contribute to innovations in the micro-sized AOCs, including discoveries of new materials and the fabrication of sophisticated morphologies and decorations on the surfaces of microparticles. Below, we discuss the novel design and functions of a few groups of micro-sized AOCs (such as Hb- and PFC-based AOCs) and others, along with the resulting improvements in circulation, oxygen-releasing profiles, and biocompatibility through illustrative examples.

4.1 Hb-based micro-sized AOCs

Owing to Hb's excellent oxygen-carrying ability, HBOCs have remained as the largest topic in either nano-sized or micro-sized AOCs for decades. The weight percent of Hb molecules in an RBC is approximately 33%; this can also act as a target for the encapsulation rate of Hb in micro-sized HBOCs.

The sources of Hb are divided into human and animal (commonly bovine and marine). The corresponding Hb products vary in terms of the oxygen capacity, affinity, stability, and other chemical properties [14]. The extracted Hb molecules are usually chemically modified to prevent protein decomposition,

which may cause toxicities to organs such as the kidneys [103]. Hb with a large molecular weight can be obtained by crosslinking and polymerization to prevent the molecular conversion from a single Hb to its dimer form [104]. To further increase the intravascular retention, the modified or crosslinked Hbs can be conjugated to or encapsulated into lipid and polymer shells to form Hb-encapsulated microparticles.

Another strategy encapsulates Hb without chemical crosslinking. To achieve effective encapsulation of Hb molecules, this strategy requires a suitable supporting material able to sustain the Hb core without affecting the oxygen transportation performances, in addition to avoiding other side effects. Phospholipids have been studied for the preparation of artificial RBCs but are very expensive. Thus, alternative biodegradable polymers have been studied for encapsulation, such as polylactide (PLA), PEG, polyethylene oxide (PEO), and PCL [105]. Moreover, amphiphilic copolymers based on combinations of these biodegradable polymers (PEO/PLA, PEO/PDB, PEG/PLA, PEG/PLC, etc.) have be used to encapsulate Hb, superoxide dismutase catalase, and methemoglobin reductase while self-assembling and avoiding protein absorption. Those diblock amphiphilic copolymers can also degrade into harmless species and form polymersomes or micelles in the body fluid with similar micellar structures and sizes as those of liposomes [106]. In view of the above advantages, new HBOC microcapsules based on amphiphilic copolymers have been developed in recent years. Sun et al. [107] encapsulated carbonylated Hb molecules into a polypeptide platform made from poly(l-lysine)-block-poly(l-phenylalanine) diblock copolymers. The core-shell microparticles had a spherical morphology with a mean diameter of approximately 4 to 5 μm; the Hb content reached 32 wt.%. Wang et al. [108] formed polymeric HBOC micelles with triblock copolymers of poly(ethylene glycol)-block-poly(acrylic acid)-block-polystyrene (PEG-b-PAA-b-PS) for photodynamic therapy. In their novel approach, the PEG-b-PAA-b-PS copolymer could self-assemble into polymeric micelles and the Hb was covalently conjugated by the carbodiimide chemistry. Then, zinc phthalocyanine was encapsulated into the HBOC micelles as a photosensitizer, resulting in a polymeric photodynamic therapy agent with an oxygen self-compensating ability.

As reported by several researchers, proteins also show potential for creating oxygen carriers owing to their longer stability, biocompatibility, biodegradability, amphipathic nature, and half-life [109]. Albumin has become another candidate for the shells of micro-sized HBOCs owing to its high affinity to hydrophobic Hb molecules [110]. For instance, Lai et al. [80] and Ohta et al. [82] used a similar strategy to fabricate bovine Hb-albumin (bHb-BAS) and human Hb – albumin (hHb-HAS) microspheres with narrow size distributions, respectively. They crosslinked bHb-BAS and hHb-HAS emulsions based on glutaraldehyde and formed mono-dispersed micro-sized HBOCs showed high resistance to metHb formation and an Hb weight ratio of 10 wt.% to 20 wt.%, respectively. Although glutaraldehyde is the most commonly used crosslinking agent, excess glutaraldehyde is toxic and requires efforts for removal. Recently, Kai et al. [62] developed micro-sized HBOCs encapsulated by genipin-linked BSA shells, yielding an optimal encapsulation rate of 40–45 wt.% for the crosslinked Hb.

Owing to the thermodynamically reversible nature of the Hb and oxygen binding reaction, the photothermal effect induced in the light-responsive particles by near-infrared irradiation makes controllable oxygen delivery possible with HBOCs. Liu et al. [111] utilized typical light-responsive particles, i.e. molybdenum disulfide quantum dots (MoS_2 QDs), to control the oxygen release from HBOCs to the targeted area in tissue. For this purpose, the Hb and MoS_2 QDs were incorporated into a gelatin methacrylate (GelMA) inverse opal (GelMA is a microcarrier gel with an ordered porous structure). Similarly, Zhang et al. [112] developed Hb and light-responsive black phosphorus quantum dots-loaded GelMA microneedles for accelerating oxygen release by controlling local temperature increases on a skin. However, the current photothermal control approach still has limitations. For example, it can only shift the binding equilibrium one-way to unbinding, i.e. it cannot suppress the oxygen release.

Notably, Wang et al. [113] recently developed a novel design for a micro-sized HBOC (Hb-HKUST −1) with porous metal-organic frameworks (MOFs), with HKUST−1 as core and Hb as the shell. Owing to the Hb-HKUST−1, this design had an eight times larger oxygen capacity than that of pure Hb and a longer oxygen retention time of approximately 16 hours. Although this new micro composite still has certain potential issues owing to its irregular shape, low deformability, and unknown stability, it may suggest the utilization of MOFs for oxygen delivery *in vitro* and *in vivo* as a new area for AOCs.

4.2 Perfluorocarbon (PFC)-based micro-sized AOCs

PFOCs are the second most investigated type of AOCs. Although the oxygen solubility of PFCs does not equal that of Hb, they are much easier to process, stabilize, and incorporate into microcapsules, owing to their chemically inert nature. An et al. [114] first developed PFC-loaded microparticles as micro-sized PFOCs by encapsulating mixed perfluorodecalin and perfluorotripropylamine droplets into a PEG diacrylate-based hydrogel. Nevertheless, the encapsulation rate of the PFC nanoemulsions in the gel microparticles was

generally very low, e.g. at 0.12%. This is because oxygen rapidly diffuses from the PFC emulsion as radicals and interferes with the UV-initiated polymerization of the hydrogel microparticles. This issue was overcome by the encapsulation of PFC molecules directly in the polymeric shell by demulsification, significantly increasing the encapsulation rate. In recent years, most studies have fabricated micro-sized PFOCs with core-shell structures by using PEG, PCL, PLC, PLGA, L-a-phosphatidylcholine, and other polymers as shell materials. Instead of simply providing stabilization using the surfactants on the droplet surface, polymer-shelled microparticles require much less surfactant and can avoid most of the surfactant molecules on the surface by centrifugation after their production. Thus, the core-shell structured PFOCs have higher biocompatibility, a longer oxygen-releasing time, and a longer shelf life. For example, Lee et al. [115] developed PCL/perfluorooctane (PCL/PFO) core-shell oxygen carriers with sizes of 100 to 500 μm and an average shell thickness of 10 μm. Under a hypoxia condition of 1% oxygen, the PCL/PFO microcapsules could release sufficient oxygen through the porous membrane for cell proliferation for t3 days (>5.0 mg/L) and were exhausted by 10 days, demonstrating the ability to prolong cell survival until the ingrowth of new blood vessels. Later, the same group seeded human bone marrow-derived mesenchymal stem cells uniformly on the same PCL/PFO system and proved a significant increase of survival in stem cells survival adhered to the microcarriers owing to their sustainable oxygen release. This research demonstrated the potential of using micro-sized AOCs to aid stem cell therapy [116].

PLC is well-known as a thermoplastic biodegradable elastomer with the potential to act as a tissue engineering scaffold (e.g. for a blood vessel, esophagus, or bladder) owing to its elastomeric properties [117–119]. Fu et al. [81] utilized PLC to produce PLC/perfluorooctyl bromide (PLC/PFOB) microcapsules called concave-shaped deformable PFC-based oxygen carriers. These showed high biocompatibility and deformability owing to their elastomer PLC shells. They passed through silicon microchannels with a width of 4.5 μm despite having sizes larger than 6 μm, i.e. similar to those of RBCs. It will become important to mimic the excellent deformability of RBCs to utilize micro-sized AOCs.

4.3 Other micro-sized AOCs

POs and OMBs are two other rising types of oxygen carriers. The utilization of solid or liquid peroxides as oxygen-generating biomaterials to facilitate tissue regeneration has risen in recent decades. This type of oxygen carrier has a unique property in that they do not require the absorption of the ambient oxygen as they work. The mechanism of oxygen generation of

a PO is straightforward; they react with water to produce hydroperoxide, which decomposes into water and oxygen. However, as water is always abundant in the environment, the reaction is very fast and uncontrolled; thus, the resultant volatile oxygen bubbles and radicals can cause cell death [120]. This problem is perfectly solved by the encapsulation of the PO particles in a hydrophobic polymer as barriers to oxygen permeation to slow down the oxygen generation rate and prolong the half-life of the release time. Therefore, PO-based, polymer-covered microparticles are frequently embedded or crosslinked in scaffolds or gels to provide sustainable oxygen to cells [65,121–123].

According to the reaction kinetics, the oxygen capacity and release rate of metal PO-loaded micro-sized AOCs are proportional to the PO concentration in the microparticle. However, a high concentration of PO could increase the formation and accumulation of intermediates such as H_2O_2 and result in undesirable cytotoxicity [124]. To eliminate the side effects owing to reactive oxygen species, catalase (an antioxidant enzyme) can be used to accelerate the decomposition of H_2O_2 [125]. For example, Mohseni-Vadeghani et al. [126] recently immobilized catalase on the surfaces of CaO_2-loaded PLLA microparticles and CaO_2/PPLA core-shell microparticles as oxygen carriers. They showed that a 2:1 weight ratio of catalase to microspheres with a catalase grafting efficiency of 17.1% was optimal for increasing the cell viability by speeding up the H_2O_2 decomposition. Furthermore, they showed different oxygen and calcium ion release profiles from the PO-filled microparticles and core-shell microparticles owing to the structural discrepancies.

OMBs are micro-sized AOCs filled with oxygen or PFC gases and coated with biomimetic and oxygen-permeable lipids. As the lipid shell is ultra-thin and can be easily deformed or destructed when subjected to ultrasound irradiation, OMBs are frequently used in ultrasound-mediated oxygen therapy. The oxygen capacity is then directly proportional to the sizes of the OMBs; it usually varies from 5 to 500 μm to according to the application and geometry of the targeted area [127]. Commonly, OMBs must be stabilized using highly concentrated surfactants, making them prone to coalescence and Oswald ripening [128]. Although OMBs can provide a high amount of oxygen in a short time by *in vivo* intravenous injection, the retention time of OMBs is generally very low (in minutes) relative to those of other micro-sized AOCs [129]. Thus, for a more sustainable oxygen supply, the lipid shells of OMBs must be strengthened to achieve a higher circulation resistance. For instance, Reusser et al. [130] utilized longer acyl-chain phospholipids as shells to increase the circulation time of the microbubbles to approximately 2 hours. Alternatively, the short circulation life of OMBs can be resolved by continuously

supplying microbubbles through the vascular system to treat hypoxia. Very recently, Vutha et al. [88] developed an OMB delivery system based on a microfluidic device to realize real-time, on-demand, and long-term intravenous oxygen delivery. They created a flow-focusing microfluidic device for producing lipidic OMBs with controlled particle sizes, tunable gas fractions, and volumetric flow rates. In their device, the initial microbubbles were broken up into microbubbles owing to a velocity-induced shear controlled according to customized nozzles and step changes. Thus, resultant OMB size could be changed in real-time by simply adjusting the liquid and gas input flow rates. These types of systems have great potential for automatically delivering oxygen on-demand, as they can be incorporated into oxygen-monitoring systems to providing on-time regulation of the production rates of the OMBs.

4.4 Comparison of current micro-sized AOCs to human RBCs (hRbcs)

Following years of development, many developers of current micro-sized AOCs are trying to mimic hRBCs to match their better performance in circulation. Some AOCs have achieved certain characteristics of hRBCs, such as their biconcave morphology, high elasticity, or high Hb loading by using alternative biomaterials, whereas others derived from RBC components (such as cell membranes) have generally become closer to original hRBCs.

In Table 1, we summarize examples of micro-sized AOCs either inspired by hRBCs or intended to mimic their properties and functionalities.

As mentioned above, the impressive deformability of RBCs enables them to fold themselves as they pass through a vascular capillary with an inner diameter smaller than their size. Mimicking such deformability is crucial and especially meaningful for the *in vivo* application of micro-sized AOCs, as the oxygen carriers must be circulated or perfused in many cases in tissue engineering. Soft microparticles mimicking the morphology of healthy RBCs with advanced biomaterials have already been manufactured in several studies on HBOCs and PFOCs. Compared with the measured Young's modulus of healthy RBCs of 4.4 ± 0.6 kPa [133], the elastic moduli of the micro-sized AOCs made of, for example, a TEGA hydrogel could reach similarly low values, such as 6.5 kPa [102]. Moreover, micro-sized oxygen carriers are often tested and have passed through silicon microchannels and recovered their shapes like RBCs. Therefore, it is believable that new materials softer than RBC membranes for AOC shells will continue to emerge with the advances in polymer science.

The oxygen capacity of an RBC is decent owing to its 33% Hb content. HBOCs or PFOCs have an ability to approach or even transcend the oxygen capacity of hRBCs, as they can also reversibly hold oxygen molecules. For micro-sized HBOCs, the highest Hb loading rate achieved so far is 70% [102]. Regarding the micro-sized PFOCs, the oxygen-carrying capacity is also linearly dependent on the amount of PFCs incorporated in the microcarrier. However, to carry the same amount of oxygen to their maximum capacity, the micro-sized PFOCs are expected to need to be oxygenated under pure oxygen owing to the oxygen partial pressure according to Henry's law.

Table 1. Micro-sized AOCs which were inspired by hRBC.

Type	Year	Size (μm)	Shape	Shell/membrane material	Fabrication technique	Note	Ref
Hemoglobin (Hb)-based oxygen carrier (HBOC)	2009	7.0	Concave	Polylactic-co-glycolic acid (PLGA)	Layer-by-layer (LBL)	The first study to develop red blood cell (RBC)-mimicking artificial oxygen carriers (AOCs)	[95]
HBOC	2012	5.6–6.3	Biconcave	Triethylene glycol acrylate (TEGA) hydrogel	"Particle Replication In Non-Wetting Templates" (PRINT)	The particles could be loaded with over 70 wt.% Hb	[102]
HBOC	2013	6.7	Biconcave	Ca(OH)$_2$	LBL	The particles could recover their shape after deformation	[98]
HBOC	2018	6.5	Biconcave	Ca(OH)$_2$	LBL	Dextran was introduced as antithrombotic coating	[99]
HBOC	2018	2.0	Ellipse	RBC membrane recombinant	Chemical crosslinking	The mean corpuscular hemoglobin of the particle reaches approximately tenfold as high as those of natural RBCs	[131]
N/A	2019	7.5	Biconcave	Pectin-based hydrogel	Electrospray	Oxygen carriers such as Hb can be encapsulated into the hydrogel system	[132]
HBOC	2020	7.5	Biconcave	RBC ghost membranes and polymer	LBL	Flexibility of the RBC–polymer replica can be well-controlled based on different coating cycles.	[97]
Peroxide (PO)	2020	5.3	Concave/ellipse	PLGA	Electrospray	Surface topography showed cytoskeleton arrangement	[66]
Perfluorocarbon (PFC)-based oxygen carrier (PFOC)	2022	8.0	Concave	Poly(lactic acid-co-ε-caprolactone) (PLC)	Shirasu-porous-glass (SPG) membrane emulsification	Young's modulus was close to that of human RBC (hRBC)	[81]

A few research studies have adopted different methods to produce RBC replicas as templates with recombined RBC membranes for loading Hb or PFCs as oxygen carriers [96,97,131]. As those particles are derived from components in natural RBCs, they can preserve the properties of RBCs to a great degree. For instance, Liu et al. [131] recently developed aggressive man-made RBCs (AmmRBCs), i.e. spindle-shaped pseudo-RBCs with a transverse length of 0.5 μm and longitudinal length of 2 μm. In spite of the smaller volume of their particles relative to that of hRBCs, the membrane of the oxygen carrier was recombined with the hRBCs' membrane, making them biocompatible and suitable for encapsulating an Hb-polydopamine (PDA) complex. As the PDA was an antioxidant for scavenging H_2O_2, the AmmRBCs shared a similarity with natural RBCs, as both contained natural antioxidative enzymes. However, the rebuilt RBCs as oxygen carriers still have limitations such as limited sources for the materials derived from donated blood, a complicated fabrication method, and relatively low yields.

In contrast to the complex bioenvironment and fragile nature of hRBCs, micro-sized AOCs based on polymeric platforms generally comprise much more simplified components with controlled resistance to metabolic transformation under severe conditions such as high temperatures and pH changes. Accordingly, they can be sterilized by means of filtration, pasteurization, and chemical cleaning, eliminating the sources of several diseases such as AIDS and hepatitis [134]. In addition, the longer storage life tremendously reduces the potential cost of using AOCs as a blood substitute. To this end, it is expected that a new generation of micro-sized AOCs based on either Hb or PFCs possessing all of the advantages of hRBCs will be proposed in the near future.

5. Potential applications of micro-sized AOCs

AOCs have proven useful as blood alternatives, but have also demonstrated significant impacts on other biomedical fields. Nano-sized AOCs have been widely studied for various applications. However, the applied research on micro-sized AOCs was not as energetic as that on nano-sized AOCs until recently. Based on the previous applied research on nano-sized AOCs, the main fields where recent RBC-inspired micro-sized AOCs have achieved success are discussed below.

5.1 Tissue engineering via perfusion cultivation system using AOCs and channeled scaffold

Providing an oxygen supply is critically important to regenerating three-dimensional tissues *in vitro*. Like natural animal tissues which obtain sufficient oxygen

from their capillary vessel networks and blood containing RBCs, scaffolds with channels and AOCs are necessary for tissue engineering. One model simulation predicted that a perfusion of PFC-based AOCs such as Oxygent® would be useful for the cultivation of cardiac tissue constructs [135].

Supplying oxygen using AOCs requires a perfusion system for the cell culture media containing the AOCs. The cell culture media containing the AOCs work like the blood in our body. Gas exchangers and pumps work like the lungs and heart in our body, respectively [136–138]. The perfusion system shown in Figure 7(a) simulates a circulation system similar to that in the body. The media containing PFC nano-sized micelles flow through the channels of scaffold in one direction. The perfusion system shown in Figure 7(b) utilizes an alternative infusion and withdrawal flow, enabling the system to be compact and easy to operate. Even the static culture shown in Figure 7(c) demonstrates the usefulness of AOCs for maintaining *ex vivo* cultures of human pancreatic slices. The corresponding researchers concluded that their technology would be useful for real-time regeneration/developmental studies in the human pancreas.

To the best of our knowledge, the AOCs applied in tissue engineering have mainly been nano-sized AOCs so far. In mechanobiology and physiology, the dynamic interactions between RBCs and endothelial cells comprise a popular topic that has not yet been sufficiently clarified [139]. Hence, we expect that the micro-sized AOCs inspired by RBCs will be applied in perfusion cultivation and contribute to regenerating more functional tissues by mimicking the interactions between RBCs and endothelial cells in tissue engineering and regenerative medicine.

5.2 Local oxygen therapies using AOCs and regenerative medicine

Hyperbaric oxygen therapy (HBOT) is the intermittent inhalation of 100% oxygen at a pressure greater than 1 atmosphere. This therapy increases the amount of oxygen dissolved in the blood by 10–20 times compared to the amount of oxygen obtained by breathing under a normal atmospheric pressure. This dissolved oxygen amount is slightly more than sufficient to meet the resting cellular requirements [140,141]. Furthermore, the dissolved oxygen does not transport itself via binding to Hb, so the HBOT can carry oxygen to the body independent of the amount of Hb or its ability to bind. Since Behnke and Shaw succeeded in the treatment of decompression sickness using hyperbaric oxygen in 1937 [142], HBOT has become a useful therapy for treating several diseases such as chronic refractory osteomyelitis, delayed radiation-induced injuries with bone necrosis, cardiac ischemia, air or gas arterial or venous emboli, and severe or
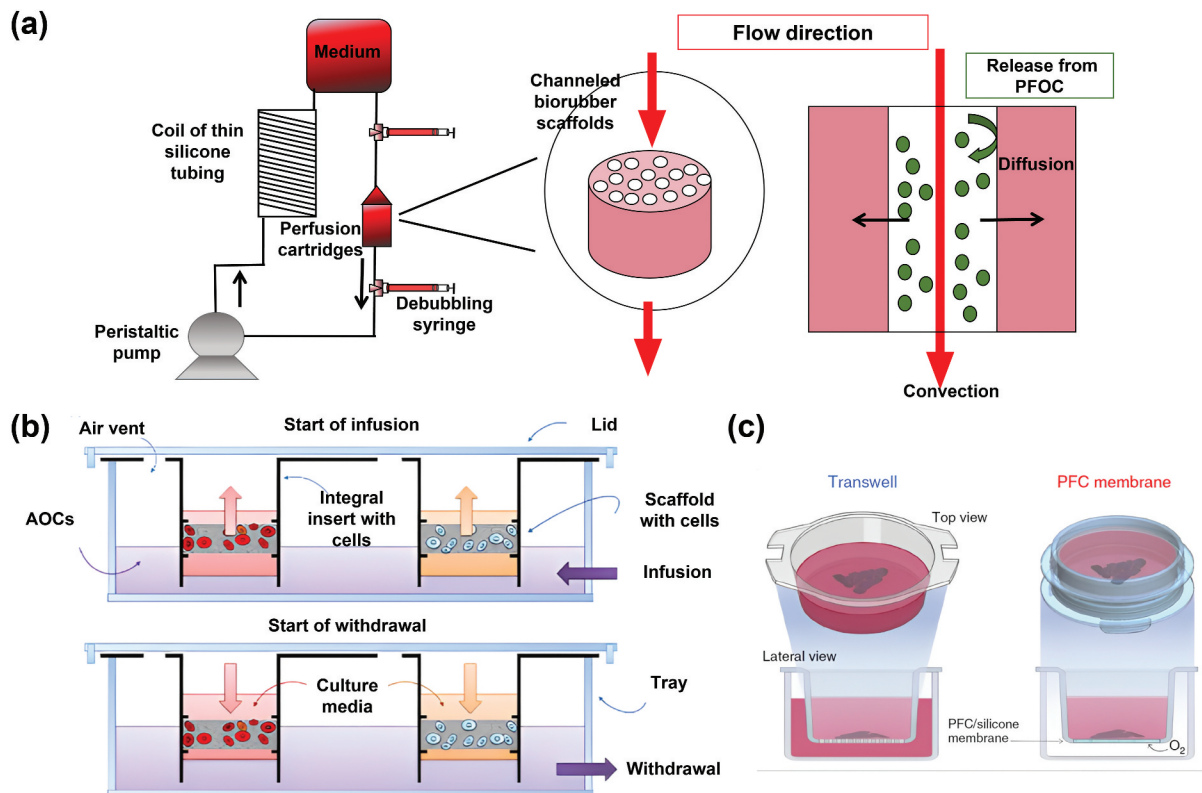
Figure 7. Culture systems using PFC-based AOCs. (a) Diagram of perfusion loop of oxygen supply with AOCs for culturing cardiac fibroblasts, adapted with permission from [136]. Copyright 2006 UPV/EHU Press (b) Diagram of PerfusionPal system operation in which dense blood substitute acts as a piston to drive the flow of medium up and down, reprinted with permission from [135]. Copyright 2020 Shoemaker et al. (c) Diagram outlining the perfluorocarbon (PFC) membrane AirHive cell culture dish compared to a transwell dish, reprinted with permission from [134]. Copyright 2020 Mirza Muhammad Fahd Qadir et al.

symptomatic carbon monoxide poisoning [140,143–147]. However, HBOT typically requires hyperbaric chambers including either mono-place chambers for accommodating a single patient at one time or multi-place chambers for accommodating multiple patients at the same pressure [145]. There is a limitation to the number of hospitals able to provide hyperbaric treatment because of the space required to set up such hyperbaric chambers. In addition, several complications from HBOTs have been described with varying degrees of seriousness [140,141]. The most frequent side effect is hyperbaric-associated middle ear barotrauma [148,149]. Recently, the possibility of using AOCs as an alternative to HBOT was investigated with various animal models. Below, we introduce recent studies using PFOCs.

Myocardial infarction is caused by the occlusion of a coronary artery, leading to the depletion of oxygen from cardiac tissue. Thus, myocardial cells in the infarcted areas under hypoxia lose their functions and undergo cell death. A sufficient and continuous oxygen supply is necessary to overcome the hypoxic conditions of damaged cardiac tissues. In 2005, Radisic et al. reported that PFC emulsions were effective in myocardial protection therapy, based on a mathematical model of oxygen distribution from the PFC and experiments on rat neonatal cardiac

tissues cultured in a medium supplemented with PFC emulsions [135]. Liu et al. showed that a single dose of a dodecafluoropentane -based perfluorocarbon emulsion could significantly reduce myocardial injury in a rat myocardial ischemia model using 99mTc-duramycin single-photon emission computed tomography imaging [150]. Furthermore, Qin et al. showed that pigment epithelium-derived factor-loaded PFC nanoemulsions reduced ischemic myocardial injuries in a rat myocardial ischemia model [151].

Peripheral nerve injury is another indication for HOBT because oxygen promotes nerve regeneration and reduces inflammatory cells. For instance, Schwann cells are involved in peripheral nerve regeneration, including myelin formation, the secretion of neurotrophins, and the production of extracellular matrix molecules. In 2013, Ma et al. revealed that perfluorotributylamine (PFTBA) with a thrombin-enriched fibrin hydrogel increased the viability of Schwann cells and the expression of regeneration-related genes under hypoxia in both 2D and 3D cultures [152]. In rats with 12-mm-sciatic nerve defects, transplantation of Schwann cells with the PFTBA hydrogel showed better sciatic nerve regeneration in comparison to a control without the hydrogel [153]. However, the time for releasing oxygen from the PFTBA hydrogel was limited to 48 hours. Later, Teng et al. modified the

PFTBA hydrogel using a coaxial electrospinning technique to prolong the oxygen supply [69]. PFTBA core-shell fibers containing PFTBA in the core and PCL-chitosan in the shell were able to extend the oxygen supply to up to 144 hours. The Schwann cells in the PFTBA core-shell fibers increased the survival and expression of regeneration-related genes under hypoxic conditions *in vitro*. Furthermore, improved axonal regeneration and remyelination by the PFTBA core-shell fibers were observed in a 17-mm rat sciatic nerve defect model.

Chronic refractory osteomyelitis and delayed radiation-induced injuries with bone necrosis are also indications for HOBT, because hypoxia inhibits bone growth and recovery. Furthermore, in the case of osteomyelitis caused by successive invasions of micro-organisms, the oxygen supply has a direct antibacterial effect and enhances the antimicrobial activity of leukocytes. Recently, it was reported that human periosteum-derived osteoblasts in PFO emulsion-loaded hollow microparticles containing PFO in the core and PCL in the shell increased cell viability and maintained the ability of osteogenic differentiation [154]. In a miniature pig staphylococcus aureus mandibular osteomyelitis model, implanted human periosteum-derived osteoblasts in PFO emulsion-loaded hollow microparticles promoted new bone formation [154,155].

Other animal studies have shown that AOCs improve cell viability and functionality in several tissues, including liver and pancreas tissues. It is expected that micro-sized AOCs will be further investigated for clinical applications for HOBT-approved diseases.

5.3 AOCs and AOCs-encapsulated scaffolds for wound dressing

Local oxygenation therapy is as effective for skin wound healing as it is for myocardial infarctions and peripheral nerve injuries. The topical Hb spray Granulox® was approved in Europe in 2012. It was reported that Granulox® improved the wound closure, wound size, pain, sloughing, and exudate levels of patients with chronic diabetic foot ulcers [156].

Wound dressings containing AOCs for excellent wound healing have been studied in previous research [111,123,157–159]. Figure 8 shows an example of oxygen-releasing hydrogel sponge, i.e. an alginate-based hydrogel embedded with H_2O_2-PLGA microspheres [123]. The sizes of the AOCs containing H_2O_2 were controlled by sieving to between 50–250 μm. Perfluorodecalin-encapsulated albumin nanoparticles were embedded in the self-healing hydrogel [159]. In another study, injectable hydrogels and microcarriers [111] were tested to be as suitable as sheet hydrogels for wound dressings [157]. Recently, molybdenum disulfide quantum dots or microalgae [158] were used as AOCs for wound dressing. In this field, AOCs with sizes larger than the sizes of RBCs can be applied, as they do not circulate or make direct contact with cells.
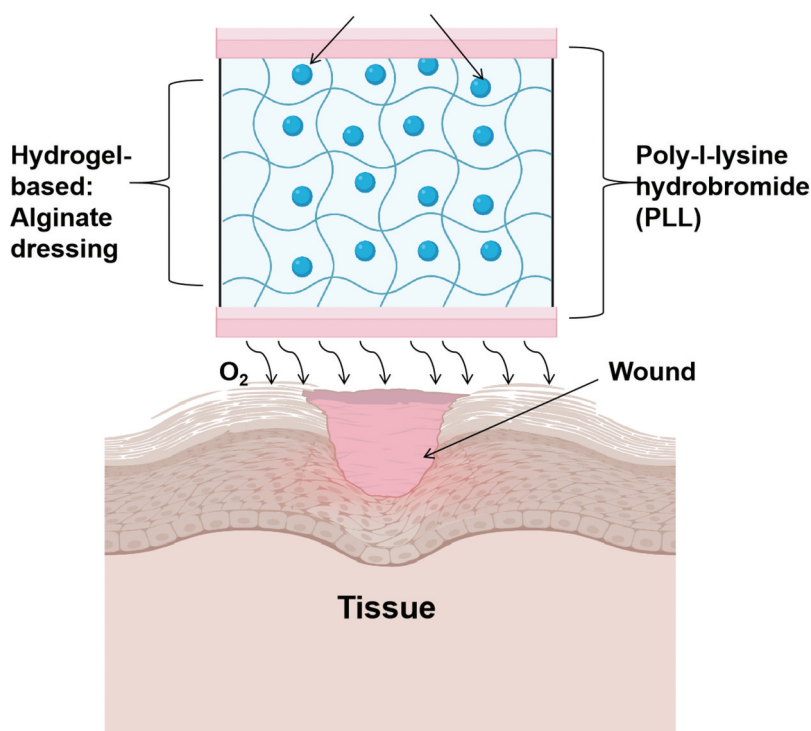
Figure 8. Oxygen releasing scaffold using micro-sized AOCs for new wound dressing, adapted with permission from [122]. Copyright 2018 Jeongyeon Choi et al.

5.4 Organ transplantation

Conventional cold storage cannot be used for many potential donor organs. For example, in general only 63% of UK deceased donor livers are transplanted. The perfusion of oxygenated blood with medications and nutrients at body temperature, called normothermic machine perfusion (NMP), is expected to solve this problem. Randomized control trials of NMP in liver transplantation have indicated its usefulness [160]. In lung transplantation, *ex vivo* normothermic perfusion (EVNP) before transplantation has been clinically studied to reassess donor lung functions [161]. HBOCs were found to show potential in several reported preclinical and clinical research studies; illustrations of certain HBOC applications are shown in Figure 9 [14]. Albumin-derived perfluorodecalin-filled nanocapsules have also applied for the EVNP of kidneys in rats, suggesting the potential of using PFOCs [162]. The functions of micro-sized AOCs for such applications have not yet been explored.

5.5 Biosensors

It is always meaningful to monitor the oxygen tension as it is closely related to fates of cells, such as their growth, differentiation, and death, tissue damage and inflammation, and cancer development [163]. RBCs are natural oxygen sensors, as oxy-hemoglobin and deoxy-hemoglobin have different visible light and infrared red-light absorption. Pulse oximeters utilize this phenomenon. The degree of RBC hemoglobin desaturation is strongly correlated to the oxygen demands of tissues. In addition, RBCs release adenosine triphosphate in response to the low local oxygen tension as they circulate in the blood vessels. Therefore, in reality, RBCs perform as both oxygen sensors and oxygen carriers, enabling them to deliver oxygen on-demand to tissues through the complex vasculature of the body [164]. For the same reason, AOCs also have the potential to be modified to monitor oxygen levels while simultaneously carrying oxygen, thereby achieving automatic site-sensing oxygen
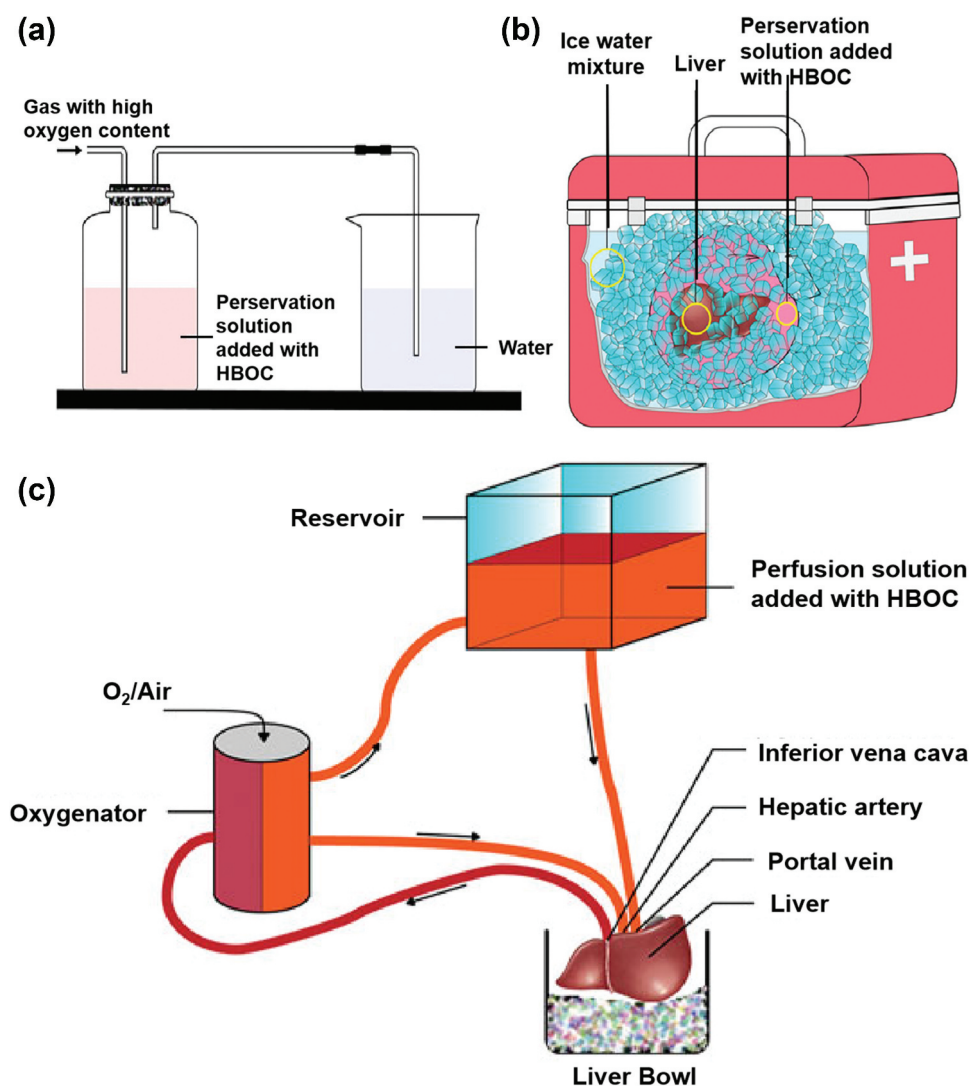
Figure 9. Application of HBOC in solid organ preservation. (a) Pre-oxygenation process of HBOC-added preservation solution. (b) Static cold preservation of isolated liver after pre-oxygenation with the HBOC-added preservation solution. (c) Mechanical perfusion of the isolated liver after pre-oxygenation. Adapted with permission from [14]. Copyright 2021 Cao et al.
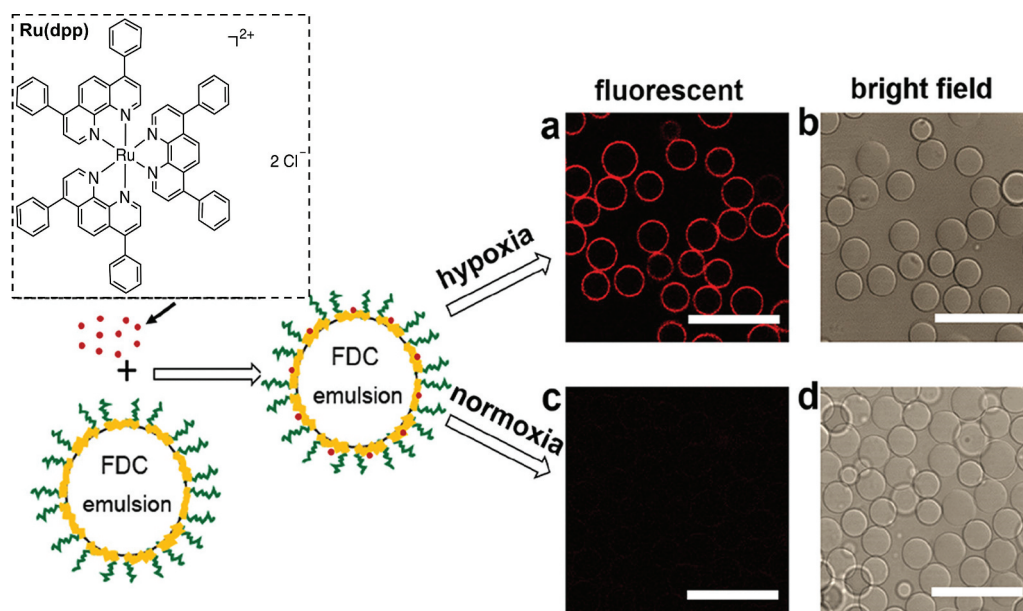
Figure 10. Functionalization process and confocal microscopic images of Ru(ddp)-loaded FDC emulsions stabilized with 2% F127 under hypoxic condition (a,b) and normoxic condition (c,d). Scale bars: 20 μm, reprinted with permission from [73]. Copyright 2019, American Chemical Society.

transfer. Liu et al. [165] recently developed chitosan/Hb microcapsules as micro-sized oxygen biosensors by immobilizing them on the surface of an Au nanoparticle-modified-glass carbon electrode. Owing to the protection of the chitosan shell, the problem of electrode fouling by direct adsorption of the Hb onto the electrode surface was evaded; moreover, the sensitivity of the oxygen biosensor was higher than when depositing Hb without a shell [166]. Previously, they prepared chitosan/Hb nanocomposites on the surface for a same oxygen-sensing purpose [167]. However, despite those nanoparticles or microparticles having functionalities for both oxygen sensing and oxygen carrying, they could not work as both simultaneously, as those particles were immobilized on the surface of the probe for enhancing the oxygen diffusion. Fu et al. [74] recently loaded Ru(dpp) nanoparticles uniformly on the surface of micro-sized perfluorodecalin emulsions as an oxygen-sensitive probe. The author showed that the micro-sized emulsions could reflect the oxygen level in the surroundings during oxygen supply to the cells *in vitro*, as shown in the confocal microscopic images in Figure 10. Theranostics is another key technology in medicine. As we described, RBCs naturally have theranostic natures for delivering and sensing oxygen. AOCs inspired by RBCs could therefore have both oxygen delivery and sensing functions in the future.

5.6 Future challenges in applying micro-sized AOCs

Despite the promising oxygen capacity of PFCs and Hb, oxygen with a high oxygen partial pressure must

still be replenished during circulation for PFOCs and HBOCs to maintain their maximum capacities. For the other micro-sized AOC types, increased efforts need to be spent on increasing the oxygen capacity or mitigating the initial oxygen burst to prolong the duration of oxygen release [168]. As most micro-sized AOCs are constructed with a core-shell structure, the properties of the shell materials are important to the behaviors of the core-shell particles during perfusion and circulation. The surface properties of micro-sized AOC particles may require modifications (such as antithrombotic coatings) for clinical use in the future. Deformability is another crucial property for micro-sized AOCs to circulate through micro-vessels; it is affected not only by the material rigidity, but also the shell thicknesses and shapes of the micro-sized AOCs. For instance, Fu et al. [81] showed that concave PFOB/PLC microparticles could pass through a silicon channel, whereas spherical PFOB/PLC microparticles with the same composition and size could not. As the practical use of micro-sized AOCs also demands large quantities of the particles, another technical challenge concerns scaling up the current techniques in the laboratory to an industrial level. Future novel biomaterials and advanced fabrication techniques are expected to address these challenges.

6. Conclusion

In recent years, HBOCs, PFCs, POs, and OMBs have been the most representative types of AOCs manufactured in microparticles or microcapsules. Despite their shared similar sizes and structural features (such as core-

shell structures), different types of AOCs had distinguishing behaviors for oxygen generation or release, and correspondingly were designed to work under different circumstances. Additional types of micro-sized HBOCs and PFOCs have recently emerged with the intention to mimic the morphology and physical properties of RBCs, which in turn would potentially provide them with favorable rheological properties. Thus, hRBCs and micro-sized AOCs were discussed in this review. The comparison between AOCs, RBCs of various species, and hRBCs provides insights for the design of novel AOCs in the future. In summary, the functionality of many current micro-sized AOCs is being improved and will continue to be improved to meet the needs of tissue engineering as biocompatible and high-performance oxygen carriers. In addition, scaled-up strategies and cost evaluations are required for the large-scale production of AOCs. It is expected that in the future micro-sized AOCs will be employed in a variety of practical uses (as are their nano-sized counterparts) in tissue engineering, blood transfusion alternatives, local oxygenation therapies, organ preservation, and so on.

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