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The definitive publisher version is available online at https://doi.org/10.1016/j.actbio.2022.05.009

Photosynthetic Microorganisms for the Oxygenation of Advanced 3D Bioprinted Tissues

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Keywords: photosynthetic microorganisms, oxygen production, 3D bioprinted tissues, aerobic metabolism, bioinks

Abstract

3D bioprinting technology has emerged as a tool that promises to revolutionize the biomedical field, including tissue engineering and regeneration. Despite major technological advancements, several challenges remain to be solved before 3D bioprinted tissues could be fully translated from the bench to the bedside. As oxygen plays a key role in aerobic metabolism, which allows energy production in the mitochondria; as a consequence, the lack of tissue oxygenation is one of the main limitations of current bioprinted tissues and organs. In order to improve tissue oxygenation, recent approaches have been established for a broad range of clinical applications, with some already applied using 3D bioprinting technologies. Among them, the incorporation of photosynthetic microorganisms, such as microalgae and cyanobacteria, is a promising approach that has been recently explored to generate chimerical plant-animal tissues where, upon light exposure, oxygen can be produced and released in a localized and controlled manner. This review will briefly summarize the state-of-the-art approaches to improve tissue oxygenation, as well as studies describing the use of photosynthetic microorganisms in 3D bioprinting technologies.

1. Introduction

3D bioprinting technology has emerged over the past 15 years as a cutting-edge approach to generate advanced bioengineered tissues and organs containing cells and hydrogels with pathophysiological features close to their *in vivo* counterparts [1–4]. For optimal cell survival and function, hydrogel compositions have been optimized to mimic the extracellular environment characteristic of the human body [3]. Bioprinted tissues are generated by formulating bioinks that are deposited within a 3D structure through the use of a 3D bioprinter to approximate the distribution and organization of cells found in the body [1,5–7]. A mixture of hydrogel and either cells in suspension (or as preformed microtissues) are extruded through the nozzle of a bioprinter in this process [8]. Hydrogel printability, durability, together with cell viability and function are commonly identified as key aspects for optimal bioprinting of tissues [9,10]. Due to their ability to integrate growth factors and cells within localized structures, 3D bioprinted tissues have been employed for both *in vitro* and *in vivo* applications. 3D printed tissues are currently studied for their potential use in organ transplantation, drug testing and disease modelling, including personalized approaches using patient-derived cells [11].

Despite the recent advances in 3D bioprinting technologies, a major challenge is the engineering of the complex pathophysiology typical of tissues and organs, including the generation of a system for the delivery of appropriate oxygen concentrations to cells [1,12,13]. For instance, hypoxia-driven cell death within 3D tissues thicker than 100-200 μm in diameter compromises viability and functionality of the constructs as it also limits waste removal [6,14], while high oxygen concentrations (hyperoxia), leads to the production of reactive oxygen species (ROS), which are primarily responsible for apoptosis *via* cytochrome c [15] and reactive nitrogen specie (RNS) production [16]. Although low levels of ROS are required for tissue homeostasis, ROS concentrations are increased under hyperoxic conditions to toxic levels that promote oxidative tissue damage and inflammation [17]. Therefore, considerations around optimal oxygen delivery for 3D bioprinting technologies are critical for the engineering of tissues and organs.

In this context, several approaches to provide oxygen within 3D bioprinted tissues have been explored. For instance, several studies have attempted to promote the formation of a physiological vascular network for optimal cell viability and function [5,9,18], as proper blood vessel formation in tissues is critical to allow cells to adapt to pathophysiological stimuli [9,19–21]. The relevance of a properly functional vascular network within 3D bioprinted tissues is proportional to the thickness of the bioengineered structure [22–24], and it is predicted that pre-vascularization of bioengineered tissues will facilitate their ability to maintain high viability and function while mimicking pathophysiological features typical of the *in vivo* tissue microenvironment. Major approaches to promote vascularization in bioengineered tissues include the use of growth factors [25], endothelial cells [26], and the development of microfluidics devices to mimic blood flow dynamics [27,28]. More recently, pre-vascularization in 3D organoids and/or bioprinted tissues has been demonstrated [10,29]. For instance, human cardiac spheroids generated from cardiac myocytes, endothelial cells, and fibroblasts have been employed as *in vitro* models for human heart pathophysiology and for the biofabrication of 3D bioprinted human heart tissues to regenerate damaged myocardium when used in bioinks containing alginate/gelatin hydrogels [30–32]. The presence of a hierarchical endothelial cell network within a cardiac spheroid has prevented the development of cell death in the center of the tissue, which correlates with improved viability and contractile function [2]. However, most approaches to date failed at fully recapitulating the required morphological, biochemical, cellular and extracellular features of the human vasculature. These include the typical hierarchical vascular network presenting small, medium and big caliber vessels, as well as cell-cell interactions favoured by *in vivo* cell ratios, the use of cells that have been cultured *in vitro* and their limitations, the absence of a functional lumen, multilayered endothelium versus monolayer endothelium found *in vivo*, followed by limited recapitulation of the extracellular matrix typical of blood vessels.

On the other hand, the use of photosynthetic microorganisms as an alternative approach for the controlled and continuous delivery of oxygen into tissues has gained increasing interest in the last decade [33,34]. This approach has been validated in several *in vitro* and *in vivo* hypoxia-related models, such as wound healing and cancer therapies [35]. Moreover, a recently published clinical trial has shown for the first time the safety of implanting microalgae in human patients for the effective treatment of full thickness skin wounds [36]. Altogether, because of their biocompatibility and photosynthetic activity, these organisms offer a promising platform to overcome hypoxia in biomedical applications.

In this review article, first we will provide insights around how oxygen delivery has been promoted via either direct or indirect oxygen release, highlighting typical features of each approach and their potential limitations for bioengineered tissue (section 2). Then, we will focus on the existing studies describing the use of photosynthetic microorganisms that have been used to improve tissue oxygenation (section 3) and how they have been combined with bioinks and cells for 3D bioprinting technologies (sections 4 and 5). Finally, we will provide insights around current challenges, future directions and potential approaches to develop oxygen-producing tissue constructs and their potential impact for direct translation from the bench to the bedside (section 6).

2. Oxygen in 3D printed tissues and current approaches for its delivery

Considerations around mechanisms regulating oxygen homeostasis are critical for the bioengineering of bioprinted tissues to maintain and grow cells in 3D [26,37]. One of the major challenges is the hypoxic microenvironment commonly present at the center of 3D bioprinted structures, which is controlled by oxygen diffusion through the tissue, limiting cell viability in any tissue thicker than 200 μm [37,38]. Oxygen concentrations within tissues and organs play several major roles during development, homeostasis as well as under pathological conditions [14,24]. These include cell metabolism and function, as well as vascular network formation and wound healing processes *via* oxygen-dependent signaling pathways [39]. Moreover, during embryonic development oxygen concentrations change in response to tissue-specific stimuli, which may differ within the same tissue under homeostatic conditions [40]. Therefore, considerations around mechanisms regulating oxygen homeostasis and a thorough understanding of optimal oxygen concentrations during tissue formation are critical for the bioengineering of tissues and organs, regardless of their application [26,37].

From the molecular point of view, hypoxic conditions trigger hypoxia-inducible factor (HIF) activation of genes regulating cell viability and function, such as hypoxia-responsive element (HRE). HIF is a heterodimeric transcription factor, consisting of an oxygen-dependent α-subunit (HIF-1α, HIF-2α and HIF-3α) and a constitutively expressed β-subunit [41]. At low oxygen concentrations (0.5-2%), HIF enables ATP production and inhibition of oxidative phosphorylation [41]. In an ischemic tissue, sustained depletion of ATP triggers cell death and necrosis [42]. Besides regulating apoptosis, HIF regulates gene expression controlling angiogenesis, erythropoiesis, extracellular matrix formation, cell proliferation, glycolysis and metabolism [41,43]. Under hypoxic conditions, HRE activates vascular endothelial growth factor (VEGF) signaling pathway *via* HIF-1α and HIF-1β [44]. A tight control of oxygen concentrations through the abovementioned molecular pathways is critical for optimal survival and growth of cells within 3D bioprinted tissues.

In an attempt to control oxygen concentrations within tissues and to enhance oxygen transportation through biomaterials, three major approaches were identified: *i)* direct oxygen delivery, *ii)* oxygen carrying materials and *iii)* oxygen generating materials (**Table 1**).

Hyperbaric oxygen therapy (HBO_2) has been extensively explored for direct oxygen delivery. This approach has been used clinically and also in an attempt to increase cellular oxygen concentration within engineered structures for several applications, such as wound healing and bone grafting [16,37,45–47]. Uncontrolled oxygen delivery via HBO_2 limits its use for tissue engineering purposes, together with the fact that does not allow for the self-renew of oxygen production and cannot be used to target a specific site [37]. Moreover, the use of HBO_2 has been associated with pulmonary damage [16] and ROS-induced cytotoxicity driven by high oxygen concentrations achieved with this method, therefore alternative approaches to better control tissue oxygenation are required [48].

Another approach for the regulation of intracellular oxygen concentrations is based on the use of oxygen carrying materials, such as perfluorocarbons (PFCs) and hemoglobin-based oxygen carriers (HbOCs) [49–54]. Thanks to their biocompatibility, PFCs-supplemented culture media have shown promising results in terms of tissue growth, cell viability and proliferation [55–58]. For this reason, they have been used for tissue engineering purposes for bone [59,60], hepatic [61,62], pancreatic [63] and neural tissues [64,65]. However, the addition of PFCs to alginate hydrogels decreases their structural stability, which is associated with poor cell function [37]. To address this challenge, addition of surfactants has been explored [66], together with the development of hydrogels functionalized with PFCs [64,67,68] and with fluorinated zeolite microparticles [69]. While PFC emulsions containing surfactants have a negative effect on cell viability, PFC microparticles can be safely used as they are not cytotoxic [37]. Although PFCs-containing biomaterials present higher oxygen diffusion rates compared to HbOCs *in vivo*, they are not able to support cellular oxygen demand longer than eight days in normoxia and three days in hypoxia, with a peak within the first 24 hours after cell seeding [37]. The use of HbOCs *in vivo* have shown to promote vascularization within three weeks [37,38]. However, despite these promising results, it use for clinical applications is limited by the development of oxidative stress-related side effects on blood pressure, pancreas, liver, kidney and brain [37,49].

A third approach to increase oxygen concentrations is based on peroxide-containing biomaterials, such as inorganic peroxide. These include calcium peroxide (CPO), sodium percarbonate (SPO), magnesium peroxides and liquid peroxides. Peroxide-containing biomaterials have been used in association with polycaprolactone (PCL) nanofibers, poly(lactic-co-glycolic acid) (PLGA) microsphere shell [70,71], polydimethylsiloxane (PDMS), as well as polyvinylpyrrolidone-hydrogen peroxide (PVP/H2O2) [26,37,38]. On the contrary to PFCs, peroxide-containing biomaterials can produce oxygen *via* the

decomposition of hydrogen peroxide without the need for an external source of oxygen. However, their sustained oxygen release can cause cytotoxic ROS production, but this does not seem to alter the structural integrity of bioengineered tissues [26,37,38].

Given the limitations typical of the abovementioned approaches, photosynthetic microorganisms have recently emerged as an alternative, offering an advanced strategy to control local oxygen concentrations. The unique ability of photosynthetic microorganisms to hydrolyze water for a sustainable oxygen supply for biomedical applications will be described in the following section.

3. Photosynthetic microorganisms used for biomedical applications

Some animals have evolved mechanisms to incorporate photosynthetic cells in their body [33,72]. In these endosymbiotic relationships, photosynthetic microorganisms feed on inorganic compounds from the host to produce organic carbon metabolites through photosynthesis, which enables animals to survive for several months in the absence of food. Additionally, photosynthetic oxygen produced by the symbiont (microalgae) allows the host to be independent from an external oxygen supply. For example, marine mollusks such as the sea slug *Elysia chlorotica*, feed on the algae *Vaucheria litorea* and incorporate its chloroplasts in the epithelial cells of their digestive system, right beneath the epidermis. This way, these sea slugs are then able to capture light energy for oxygen production and autotrophic carbon dioxide fixation. As a result, the sea slug is able to sustain itself for at least eight months when provided with only light and a source of carbon dioxide [73–75]. Marine cnidarians have also evolved endosymbiotic behaviors in nature. A well-known example is *Hydra viridissima*, which incorporates a microalgae called *Chlorella sp.*, acquiring tolerance to starvation [76]. This symbiotic relationship has been also observed and described in vertebrate animals: the salamander *Ambystoma maculatum* stably incorporated the alga *Oophila amblystomatis* inside its embryos, to increase oxygen in egg capsules and enhance embryonic growth and development [77–79].

As previously described, oxygen starvation in tissues triggers cell death, which represents a major issue in several medical and biomedical areas, including tissue engineering [38]. Based on the existing symbiotic relationships mentioned above, the use of photosynthetic organisms to overcome hypoxia in thick tissues by locally increasing oxygen tension has been proposed over the last decades, and applied to numerous medical fields such as organ transplantation, heart ischemia and wound healing [33,80].

Organs used for transplantation are typically deprived of oxygen, which leads to tissue damage, limiting its preservation time and clinical success [81]. As described above, the use of artificial oxygen carriers such as hemoglobin-based PFCs has been widely investigated; however, their stability and toxicity in humans is still an ongoing issue [82]. Alternatively, the use of photosynthetic microorganisms that can constantly and controllably deliver oxygen to harvested organs has been proposed by several groups [33,38]. Pancreatic islet transplantation has been used to replace and restore *β*-cell function in diabetic patients [83]. However, their vascular isolation when subjected to transplantation results in severe ischemia, hypoxia and dysfunction. To overcome this issue, co-encapsulated murine pancreatic islets with the microalgae *Chlorella sorokiniana* have been successfully achieved [84]. Upon illumination, islets increased insulin response to glucose, which was not observed in the control groups, supporting the importance of oxygen availability for optimal organ function. In another work, Yamaoka *et al.* [85], studied the preservation of harvested rat pancreatic tissues using a suspension of microalgae *Chlorella vulgaris*, which upon illumination delivered oxygen to the organ through a gas permeable pouch. In this study, only rats transplanted with photosynthetically-assisted organs survived for over a week, while other animals transplanted with traditional cold-preserved pancreas died within a few hours. Moreover, the first generation of perfusable photosynthetic solutions for organ preservation has been recently described, showing intravascular distribution in isolated pig kidneys, and supporting the metabolic oxygen requirements of zebrafish larvae and rat kidney slices [86]. In addition, recently published works described the ability of photosynthetic microorganisms to sustain brain functionality by either transcardial injection of tadpoles [87], or in isolated rat brain slices in suspension [88].

Photosynthetic microorganisms have also been recently used as oxygen-generating systems to decrease tumor hypoxia in mice, enhancing radiotherapy efficacy in the treatment of cancer. Photosynthetic cells such as *C. vulgaris* have been used for this therapeutic approach, and coated with erythrocyte membrane [89] or calcium phosphate [90] to form immunocompatible biomimetic systems. Coated *C. vulgaris* were delivered to the tumor site in mice and illuminated with red light to induce photosynthesis. Their photosynthesis increased local oxygen levels, which in combination with radiotherapy, effectively

prevented tumor growth *in vivo*. Another group combined *C. vulgaris* with a high-oxygen-solubility medium containing PFCs, to enrich oxygen concentration around the photosensitizer. With this approach, the photodynamic therapy was greatly increased both *in vitro* and *in vivo* [91]. Photodynamic therapy has also been proved to be successful *in vivo* with other photosynthetic organisms, such as cyanobacteria *Synechococcus elongatus* in combination with two-dimensional black phosphorus nanosheets [92] or photosynthetic bacteria (not specified strain) [93]. Moreover, this same cyanobacteria strain was recently used to create a biohybrid microorganism-based sonosensitizer in order to augment the therapeutic efficiency of sonodynamic tumor therapy, approach that was validated both *in vitro* and *in vivo* [94].

Photosynthetic therapies have also shown their potential in cardiac applications [80]. Cyanobacteria *S. elongatus* has been evaluated to protect the myocardium from acute ischemia through photosynthesis [95]. Intramyocardial injection of *S. elongatus* in ischemic hearts of immunocompetent rats was performed either under light exposure or in the dark. A control group received saline alone. Animals treated with *S. elongatus* under light exposure presented a nearly 25-fold increase in oxygenation levels after cardiac ischemia, rescuing the myocardium from acute ischemia. In an effort to generate a symbiotic relationship between cardiac mammalian cells and microalgae in *in vitro* conditions, bioengineered 3D cardiac tissues have been created [96]. In this study, a symbiotic construct composed of rat cardiomyocytes and *Chlorococcum littorale* promoted oxygen delivery to mammalian cells, while these released metabolites and waste products which were reused by algae. This led to the generation of cardiac tissues of approximately 160 µm thickness.

The incorporation of photosynthetic cells in 3D scaffolds has also been described in the field of tissue engineering and regeneration. Aiming to provide oxygen to the surrounding cells or tissues, photosynthetic biomaterials were first introduced by Hopfner *et al*. [97], who seeded microalgae *Chlamydomona reinhardtii* into commercially available collagen scaffolds, and demonstrated the ability to decrease tissue hypoxia by local photosynthesis *in vitro.* The same group went one step further and applied this novel approach to an *in vivo* model, by implanting photosynthetic scaffolds in athymic nude mice [98]. After illumination of the implanted scaffolds for up to five days to promote photosynthesis and therefore oxygen production, the defect area was highly vascularized. Moreover, these photosynthetic microorganisms have been demonstrated to be safe *in vitro* [97] and in immune competent murine models *in vivo* [99]. This approach was also used for the treatment of chronic diabetic wounds *in vivo* [100], where a patch containing *S. elongatus* cyanobacteria encapsulated in alginate beads was implanted in chronic diabetic mice wounds. Upon red light irradiation, oxygen penetration in wounds was much more efficient than commonly used topical gaseous oxygen treatment, which promoted wound healing and angiogenesis without triggering any inflammatory response in mice. Finally, Obaid *et al.* [36], have recently shown that the implantations of photosynthetic biomaterials, containing large numbers of microalga *C. reinhardtii*, is safe for human patients, allowing tissue regeneration in full-thickness skin wounds.

In order to release recombinant bioactive molecules in addition to oxygen, genetic modification of photosynthetic microorganisms such as cyanobacteria and microalgae have been also investigated and described for tissue engineering purposes [33]. When transgenic microalgae *C. reinhardtii* were seeded in collagen scaffolds, they were used to release oxygen and recombinant human vascular endothelial growth factor (VEGF) *in vitro* and *in vivo* [99]. More recently, in an effort to use photosynthetic microorganisms to promote lymphangiogenesis, the same research group used genetically engineered *Synechococcus sp.* to produce hyaluronic acid, aiming to create lymphangiogenic photosynthetic scaffolds for dermal regeneration *in vitro* under hypoxic conditions [101]. In the same line, Centeno-Cerdas *et al*. [39], developed photosynthetic sutures with genetically modified microalgae *C. reinhardtii* for the potential local delivery of oxygen and human VEGF, b-FGF and SDF-1α in wounds after surgical closure.

As described above, the concept of promoting oxygen delivery through the induction of a local symbiotic relationship with photosynthetic microorganisms represents a promising approach that could be applied in several medical fields, including the generation of complex 3D bioprinted tissue constructs.

4. Methods for the bioprinting of 3D photosynthetic microorganisms

3D bioprinting techniques are mainly classified in extrusion-based, inkjet-based, stereolithography and laser-assisted printing [102]. While inkjet and laser-assisted printing offer very high resolution, these methods have speed limitations and are less explored compared to extrusion-based bioprinting.

Stereolithography-based techniques require large volumes and therefore high cell numbers to achieve the desired cell density, and is furthermore only compatible with photo-crosslinkable materials. In extrusion-based printing, cells within bioinks are subject to mechanical stress. One of the major advantages of extrusion-based bioprinting is the ability to print at high cell densities, with shear thinning polymers preferred to protect cell viability during the extrusion process. Moreover, this technique is compatible with a wide range of materials, crosslinking methods, and allows the adaptation of different parameters, such as needle diameter, extrusion rate or temperature, depending on the mechanical properties of the bioink [103]. Not surprisingly, extrusion-based method has been more widely explored for the 3D bioprinting of photosynthetic materials. In the last decade, an increasing number of studies have focused on the 3D bioprinting of materials by combining extrudable bioinks and a wide range of photosynthetic microorganisms, including: i) microalgae, such as *C. reinhardtii* [23,104–106]*, Platymonas* sp. [107]*, Chlorella sorokiniana* [105,108], *Symbiodinium* sp. *Marinichlorella kaistiae KAS60* [109], *C. vulgaris* [110]*,* and *Chlorella pyrenoidosa* [111]*;* ii) cyanobacteria species, such as *Synechocystis* sp. [112]; or even plant cells, such as *Ocimum basilicum* [113] (**Table 2**). Because these photosynthetic microorganisms and cells require illumination for optimal light-stimulated photosynthesis, the bioinks used in these studies correspond to transparent materials, including alginate [23,104–107,111–113], gelatin [23,109,111] or agarose [113] among others.

Moreover, depending on the material as well as the application, diverse nozzle sizes have been used (from 0.25 mm [105] to 3 mm [108]), as well as different pressures (from 0.8 bar [104] to 8 bar [108]) and printing speeds (from 1.5 mm/s [110] to 70 mm/s [23]). The preferred technique for the polymerization of the photosynthetic bioinks has been immersion in a crosslinking solution of calcium chloride ($CaCl_2$), in order to minimize cell damage (see Table 2). The use of UV light (365 nm) has also been described by a couple of groups [23,111], however, only one of these studies assessed the effect of UV exposure on cell viability [23]. In this case, an alginate bioink containing microalgae was crosslinked by immersion in $CaCl_2$, which was further embedded in gelatin methacryloyl (GelMA) and exposed to UV light for polymerization. The effect of the UV light on cell viability was assessed by applying different UV intensities for 40 seconds and further characterizing cell growth and chlorophyll content. Their results indicated that UV light used did not have adverse effects on cells, which could be explained by the fact that microalgae were protected within the alginate and the short exposure time. Another study combining a GelMA structure and a microalgae-containing bioink developed a photopolymerization system using 405 nm blue light to avoid UV damage to the algae [109], and, interestingly added yellow food colouring to limit the penetration of 405 nm light into the bioink.

5. Photosynthetic materials for 3D bioprinting and their *in vitro*, *in vivo* and other non-biomedical applications

The concept of “green bioprinting” was introduced for the first time by Krujatz *et al*. [105]. In this study, growth and viability of *C. reinhardtii* and *C. sorokiniana* were evaluated in 3D printed alginate structures in order to study the optimal culture conditions for immobilized microalgae, showing that cell viability was directly influenced by length of exposure to light. Additionally, immobilized microalgae within the 3D scaffolds presented the highest viability and most stable metabolic activity compared to suspension cultures, even under non optimal temperatures [105]. Further, using a two-channel plotting method, the same research group went one step further and bioprinted and co-cultured alginate scaffolds containing *C. reinhardtii* and human sarcoma cells, showing viability of both cell types for 24 hours [104]. This study represented a substantial step in the bioprinting of symbiotic tissues, where the coculture of photosynthetic cells with human cells could potentially enable a controlled delivery of oxygen without the need of external supply in a single 3D structure.

Based on this work and on the already mentioned study described by Haraguchi *et al.* [22], where thicker 3D constructs could be fabricated by combining mammalian cells and microalgae, Maharjan *et al.* [23], described the 3D fabrication of perfusable vascularized tissue constructs using *C. reinhardtii*. In this work, microalgae were used as a sustainable bionic oxygen generator to enhance the function of bioengineered tissue constructs *in vitro* [23] (**Figure 2**). Microalgae were encapsulated in a temporary cellulose-based bioink with predesigned geometries, which after printing were embedded in a GelMA-based hydrogel construct harbouring hepatic cells. Photosynthesis of the microalgae significantly improved the viability and functionality of human cells, while also reducing hypoxia-driven response, as measured by the expression of HIF-1α. Taking the study one step further, microalgae in cellulose constructs were then

enzymatically dissolved to create interconnected microchannels, which were subsequently endothelialized, generating biologically relevant vascularized tissues. This study describes the development of a temporary bioink containing photosynthetic microorganisms to allow oxygenation of 3D constructs, which after enzymatic degradation formed a vascularized construct, representing an unprecedented progress in the successful 3D bioengineering of viable and functional tissues [23].

Recently, a study described the *in situ* 3D bioprinting of photosynthetic material for the treatment of wounds [143]. Microalgae-laden hollow fibers were created by combining an outer bioink phase with an inner $CaCl_2$ phase. The bioink consisted of GelMA and alginate containing microalgae *C. pyrenoidosa*. Alginate crosslinked in the extrusion process, and GelMA polymerization was subsequently induced by UV irradiation. This construct allowed microalgae growth for at least seven days, and reduced cell hypoxia and accelerated wound closure *in vitro.* Moreover, these photosynthetic materials were 3D bioprinted *in situ* in a diabetic wound mouse model, in order to study the *in vivo* wound healing potential. Constructs were illuminated for only two hours every three days, during 15 days total, and compared to control groups including non-illumination of the material. An increase in angiogenesis, collagen synthesis and wound closure was observed 15 days after the application when compared to the control groups. As photosynthetic cells produce oxygen under illumination, but consume it in the dark, further discussion could be done to explain how dark conditions did not result in a wound healing impairment considering hypoxia being one of its main causes, at least compared to the control group without photosynthetic cells.

Besides tissue engineering applications, other studies have described the 3D bioprinting of photosynthetic constructs for environmental or industrial applications [109, 112, 113]. However, all of these studies are highlighted in this section because they may have great importance in the translation of 3D bioprinting of photosynthetic materials, as they unlock technical details such printing parameters, materials and crosslinking methods which are biocompatible with photosynthetic microorganisms.

In terms of industrial applications, 3D bioprinting of symbiotic photosynthetic relationships was implemented to fabricate bionic coral tissues, describing an optimized photon augmentation system to enhance microalgal light absorption and growth, which could have important implications for bioenergy production, as well as other metabolic bioproducts [109]. For the fabrication of these constructs, photopolymerizable gelatin-methacrylate hydrogel, cellulose derived nanocrystals and microalgae *Symbiodinium* sp. were used, while parameters such as printability, cell survival and optical performance were optimized to support cell growth and photosynthetic activity. This research group was able to mimic both functional and structural parameters of the coral-algae symbiosis, and demonstrated that reached microalgae densities were significantly higher in the coral structures than in standard liquid growth culture. Another study demonstrated the fabrication of plant-cell laden hydrogel construct, a concept that had not been previously described. Here, isolated basil cells were embedded and extruded in a hydrogel blend composed of alginate, agarose and methylcellulose, presenting high viability and metabolic activity after extrusion and crosslinking [113]. Generation of bioenergy or sustainable energy has been described by Liu *et al.* [112], where a biological photovoltaic device was created by 3D printing a biofilm of *Synechocystis sp* over a layer of heterotrophic bacteria. Cyanobacteria were encapsulated in an alginate hydrogel and further crosslinked by immersion in $CaCl_2$. This device was able to continuously generate electricity from the symbiosis of both microorganisms: heterotrophic bacterial respiration supplied by the cyanobacterial photosynthesis was used for power generation.

To date, most of the published studies around 3D printed photosynthetic microorganisms have reported their viability and durability for at most one month. In the line of environmental applications, Zhao *et al*. [107] designed a silk protein-based hydrogel for long-term hosting of living microalgae. In this work they were able to observe photosynthetic activity by means of oxygen measurements for at least 90 days, demonstrating the stability and long-term functionality of the construct, proposing potential environmental applications. Moreover, a study recently described the use of extruded algae-laden hydrogels for large scale environmental applications including bioremediation [108]. Eight different alginate-based hydrogel samples with varying polymers and water percentages containing *C. sorokiniana* were pneumatically extruded and characterized. Rheology studies were performed to establish material printability and compatibility for large scale printing, and viability of microalgae were studied in all hydrogel sets for up to 21 days. In another work, a cost-effective bioprinting approach for the fabrication of a resilient photosynthetic living material was described [106]. Alginate hydrogels containing *C. reinhardtii* were bioprinted onto a cellulose substrate, which conferred mechanical robustness against physical distortion. The photosynthetic cells were able to grow for at least four weeks within the material, with potential

applications such as for the generation of artificial leaves, bio-garments, or adhesive labels [106]. Similarly, another study has also used microalgae *C. vulgaris* to create eco-friendly 3D printing ink that was processable under ambient conditions in the form of an emulsion [110].

Altogether, several studies have recently shown the feasibility of bioprinting photosynthetic microorganisms for different applications. However, there are still several important challenges to overcome before the implementation of this concept as a successful approach in 3D bioprinted tissues.

6. Challenges and perspectives

The term holobiont means “whole unit of life”, which addresses the concept that the host and its associated microorganisms exist as an integral unit, where both contribute to its final phenotype [114]. A similar concept could be explored to develop alternative strategies towards the biofabrication of functional tissues, integrating photosynthetic microorganisms and human cells. In this context, a better understanding of the already established photosynthetic capabilities in symbiotic animals could help to address this challenge, especially in issues concerning biocompatibility, as well as molecular strategies to further reach a state of metabolic coupling. Amongst the vast array of holobionts, corals may represent an excellent research model, as they are well studied, highly diverse, and rather simple compared to other symbiotic animals [115]. Moreover, corals form a close association with a wide range of diverse microorganisms, including photosynthetic microalgae. This species-specific relationship suggests the existence of recognition and tolerance mechanisms, as well as an already optimized microenvironment that could potentially be mimicked and implemented for 3D bioprinting approaches, hence supporting photosynthesis together with cell growth and function of the host.

As shown in **Table 2**, only few photosynthetic species have been described for 3D printed biomedical approaches. Therefore, a critical challenge in this field is the need to explore the immense biodiversity that is present among cyanobacteriae and microalgae, to ensure the best possible fit for the optimal conditions to build and grow different 3D bioprinted constructs, which varies depending on the tissue of choice and their applications. It is also important to consider that having a functional vasculature not only serves to provide oxygen and nutrients to tissues, but also removes metabolic waste and other toxic molecules, which is critical in a 3D bioprinted construct [2,14]. Thus, another important challenge in this field is to generate alternative strategies where metabolic byproducts could be metabolized by each other. In fact, the most plausible of these metabolic couplings may be related to the accumulation of carbon dioxide released as byproduct of the mitochondrial respiration, which could be removed from the tissue microenvironment by its reduction in the Calvin cycle that is present in photosynthetic organisms.

As indicated by its name, photons are the energy that power the photosynthetic machinery, as consequence, the development of photosynthetic therapies requires the establishment of alternative and reliable illumination technologies to optimize the wavelength, powering, and heat release from the illumination source. In the context of 3D printing, the design of transparent biocompatible inks is a crucial issue to allow light penetration into the printed construct. Interestingly, some commonly used biomaterials already fulfill this important requirement. For instance, alginate is a widely used transparent and biocompatible hydrogel, and has been already explored to print photosynthetic microorganisms [104,105]. Similarly, collagen and gelatin also allows light penetration, being widely used for 3D bioprinting applications and have been modified for the generation of photoactivated polymers [116]. In particular, both ColMA and GelMa have been used to 3D bioprint tissue using light activation [135]. This is based on the use of photoactivated polymers (*e.g.*, ColMA, GelMA, PEGDA) together with photoinitiatiors (*e.g.,* Irgacure and LAP) which use either a 350 (UV light) or a 405 (blue light) nm light source. However, exposure to an additional light source for the activation of photosynthetic cells may interfere with the photoactivation of bioinks containing GelMA and ColMa hydrogels, which would require additional optimization of protocols used. UV light can potentially decrease cell viability, whereas 405 blue light source could be used, but it does activate the photosynthesis, which may require further considerations in case a temporal and localized oxygen delivery is required. Therefore, before its clinical success, the development of novel illumination devices and the optimization of transparent printable materials are required.

Overall, the use of photosynthetic microorganisms presents the unique opportunity to better control oxygen delivery, similar to the tight regulation occurring in embryonic development. Throughout the

review article we highlighted three major ways to do this via the use of photosynthetic microorganisms: *i)* by controling the light conditions/exposure; *ii)* by distributing within bioprinted tissues somatic cells and algae according to a specific desired pattern; *iii)* by developing and using GMOs that promote oxygen delivery on demand.

Within the illumination context, as most human cells are located in dark areas within the body, there is a chance that a potential phototoxic effect would arise as a side effect of the established illumination protocols. This would require more research to better understand how dark-living human cells could handle intense illumination settings for photosynthetic stimulation. In contrast, it is clear that photosynthetic organisms are equipped with several molecular tools for ROS protection. In fact, it is well described that enzymatic and non-enzymatic mechanisms allow plants to handle extreme oxidative loads, especially under high illumination conditions [117], which is something that could be mimicked, induced or implemented for dark-living cells but, in case it is needed, the optimal photo-protective mechanisms required for bioprinted tissues remain to be identified.

On the other hand, the required light access for algae within internal tissues remains a challenge. Although photosynthetic microorganisms cultivated in photobioreactors represent a promising approach to generating bio-based products, excessive cell density prevents light access within dense cell suspensions decreasing biomass production. Thus, genetic mutations of microalgae to minimise light absorption have shown encouraging results [33].

As previously described in this review, the use of gene modified photosynthetic cells represent a promising approach to promote regeneration and tissue formation. For instance, in addition to oxygen, genetically modified microorganisms could release other bioactive molecules in a local and controlled manner, representing several advantages compared to other similar approaches. The use of bioactive molecules has several drawbacks including short half-life of the molecules *in situ*, difficulties for local delivery, especially in the absence of vascular supply, and potentially high costs for massive implementation. Gene modified human cells could also provide such fresh bioactive molecules *in situ*. However, despite important advances in gene therapy, several issues need to be addressed before its therapeutic implementation [118], most of them also relevant issue to be solved in the context of photosynthetic therapies.

As summarized in **Figure 1**, some of the challenges described above should be considered as potential advantages compared to current oxygen delivering materials, providing a biocompatible platform for the controlled and localised oxygen production and recombinant bioactive molecules release. Hence, having a potential impact on the bioengineering of advanced *in vitro* models, where the lack of appropriate oxygenation represents a major drawback [5]. In addition, by overcoming the limit in oxygen diffusion in the middle of tissue constructs [9], photosynthetic bio-printing could contribute by generating larger tissues for transplantation and promote survival *in vivo* after transplantation. However, such highly relevant issue has not yet been described and remain to be elucidated in further research.

Other critical considerations should focus on challenges for the clinical translation of green bioprinting to from the bench to the bedside. Among them, considerations around regulatory bodies (*e.g.*, FDA, EU, etc.,) are crucial, as the implantation of microalgae and cyanobacteria as potential therapeutic agents have not been fully validated yet. Due to the novelty of this concept, there is a lack of existing clinical data describing potential long term effects of this approach, including triggering certain allergic reactions or even the transfer of novel pathogens. However, it is predicted that following preclinical and clinical validations, green bioprinting might represent an opportunity for clinical application. To date, there are first reports about the first transplantation of photosynthetic cells in patients [36]. Additional considerations are required in case of using of genetically modified photosynthetic microorganisms, which may help to further control the oxygen delivery and additional bioactive recombinant proteins or peptides *in situ* required for the specific application.

Finally, it is important to highlight that a key challenge for the future success of bioprinting approaches using photosynthetic cells will fully rely on our ability to foster interdisciplinary knowledge, bringing together different fields of knowledge with the common goal to establish human photosynthesis as a novel therapeutic approach for tissue engineering and regeneration, as well as for other physiopathological conditions where the lack of appropriate oxygenation plays a fundamental role.

6. Acknowledgements

This work was supported by a UTS Seed Funding, Catholic Archdiocese of Sydney Grant for Adult Stem Cell Research and a University of Sydney/Sydney Medical School Foundation Cardiothoracic Surgery Research Grant (to CG) and ANID-FONDECYT 1200280 Grant (to JTE).

7. Ethical Statement

Not applicable

8. Conflicts of interest

The authors declare no conflict of interest.

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Figures

Figure 1

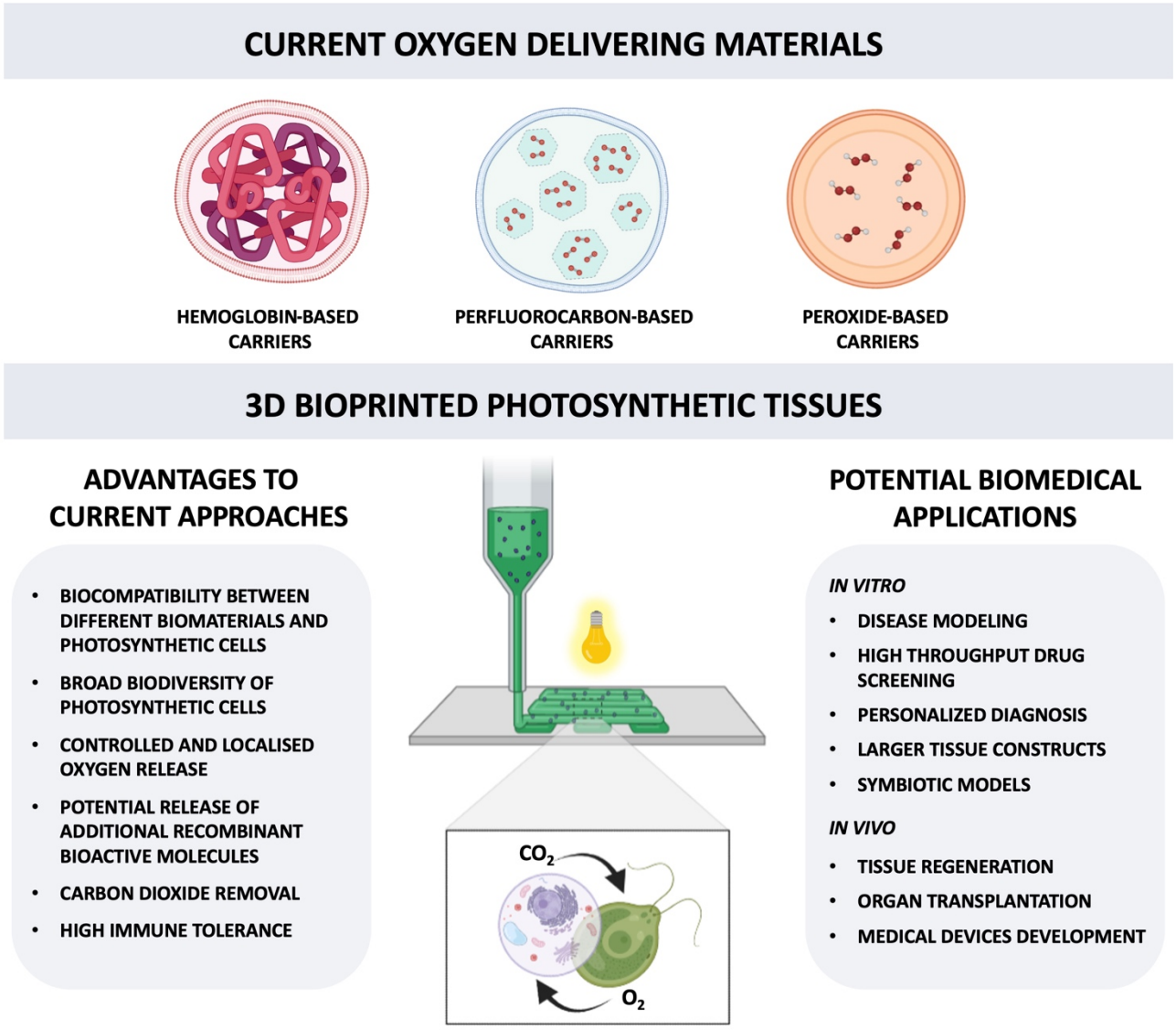
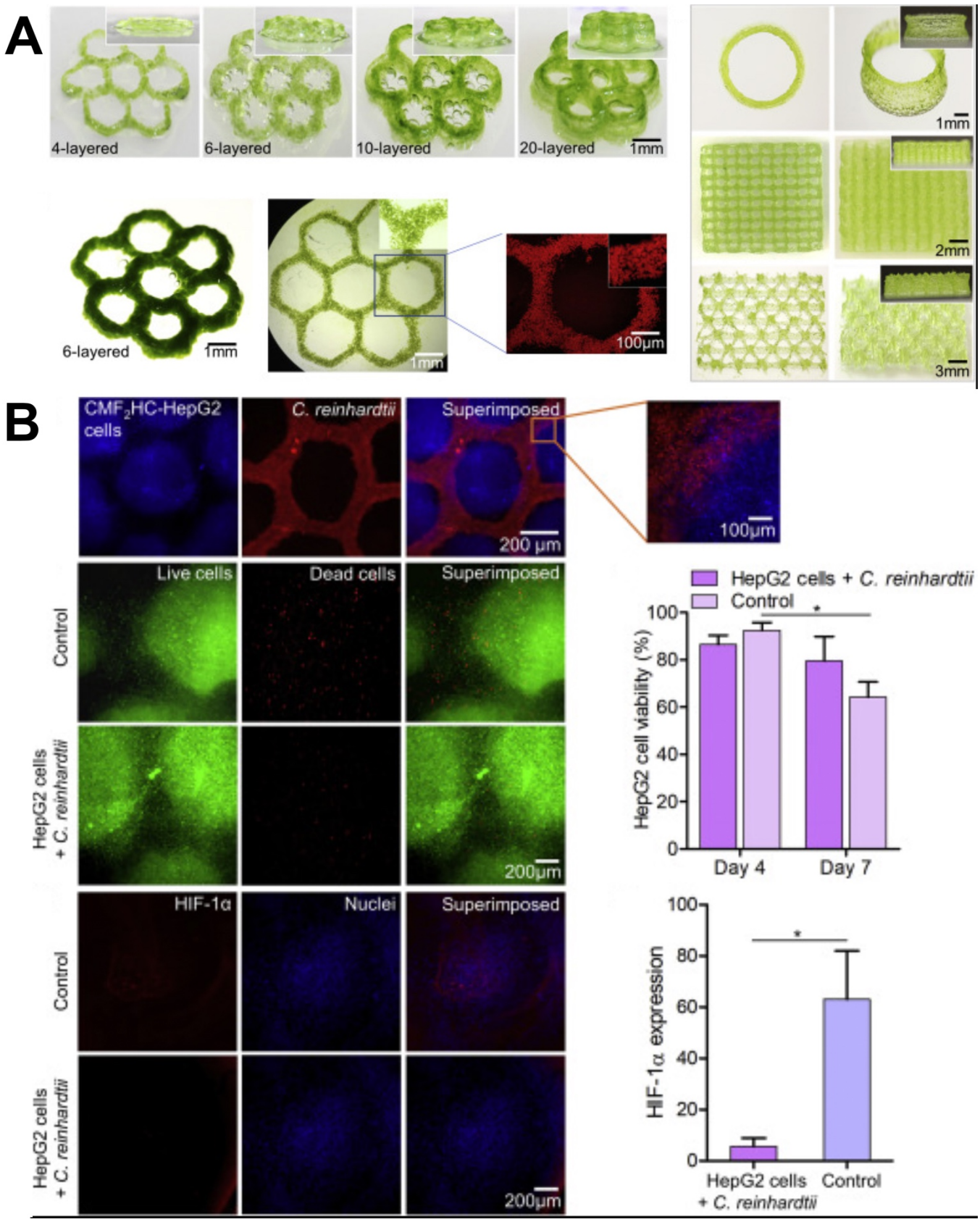


Figure 2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B
CMF2HC-HepG2 cells
C. reinhardtii
Superimposed
200 µm
100µm
Live cells
Dead cells
Superimposed
Control
HepG2 cells + C. reinhardtii
200µm
HepG2 cells + C. reinhardtii
Control
HepG2 cell viability (%)
Day 4
Day 7
HIF-1α
Nuclei
Superimposed
Control
HepG2 cells + C. reinhardtii
200µm
HIF-1α expression
HepG2 cells + C. reinhardtii
Control