A Review on Bioinks and their Application in Plant Bioprinting

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Abstract: In recent years, the characterization and fabrication methods concerning new bioinks have received much attention, largely because the absence of bioprintable materials has been identified as one of the most rudimentary challenges for rapid advancement in the field of three-dimensional (3D) printing. Bioinks for printing mammalian organs have been rapidly produced, but bioinks in the field of plant science remain sparse. Thus, 3D fabrication of plant parts is still in its infancy due to the lack of appropriate bioink materials, and aside from that, the difficulty in recreating sophisticated microarchitectures that accurately and safely mimic natural biological activities is a concern. Therefore, this review article is designed to emphasize the significance of bioinks and their applications in plant bioprinting.

Keywords: 3D bioprinting; Bioinks; Titanium dioxide; Decellularized extracellular matrix; Plant bioprinting

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1. Introduction

Three-dimensional (3D) bioprinting, also known as additive manufacturing (AM), is a rapidly evolving field, with a focus on fabricating organ and tissue constructs by layering organic materials, living cells, and biochemicals according to a given digital model[1-2]. This method is a more precise means of recreating complex structures compared to other similar technologies[3]. Over the past 15 years, progress in 3D printing technologies has produced novel technologies such as biplotting, extrusion-based bioprinting, stereolithography, inkjet-based bioprinting, fused deposition modeling, and laser-based bioprinting[4]. However, these techniques still have limitations regarding cell viability, long-term functionality, and accurate process parameters (Table 1)[2]. Bioinks constitute a significant element of all bioprinting procedures, as they are used to create the final shapes of the desired tissue structures, and they are stabilized or cross-linked during or immediately after bioprinting[5]. The insufficiency of bioinks (bioprintable materials) is a significant stumbling block in the domain of 3D bioprinting for printing tissues[6]. Bioinks are basically biomaterial solutions containing living cells and are essential components in bioprinting[2]. Bioprintability, non-toxicity, insolubility in cell culture medium, visco-elasticity, high mechanical integrity and stability, the ability to stimulate cell adhesion, and biodegradability at a steady rate are all necessary features of biomaterials for enabling high-quality tissue regeneration. Non-immunogenicity and permeability of nutrients and gases are also critical characteristics of an ideal bioink (Figure 1). In 2003, the term “bioink” and the phrase “biopaper” were first used in the context of organ printing[7,8]. The original plan was to create (or possibly print) a biopaper (hydrogel) and then bioprint it using living cells or tissue spheroids as the bioink[9]. Bioink was first used to describe a biological component that was inserted in 3D on or within a hydrogel.

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A variety of materials and combinations of materials can be utilized as bioink, but among them, the most popular and promising are hydrogels (water-based gels), which are biocompatible and have extracellular matrix (ECM)-like qualities. Hydrogels are the most commonly utilized biomaterials in 3D bioprinting on account of their modifiable chemical structures, biodegradable qualities, ability to hold live cells, customizable mechanical features, and ability to generate acceptable resolution during printing. Hydrogels are natural biomaterials, synthetic biomaterials materials, or a combination of the two that exploits the benefits of both. Bioink hydrogels made with natural biomaterials promote cell development by mimicking the natural ECM, self-assembling, and enabling biocompatibility; however, within the in vivo atmosphere, natural hydrogels may be structurally insufficient, unstable, or inconsistent, which can lead to printing difficulties, flimsy tissue architecture, and insufficient structural support for cells. On the other hand, hydrogels made with synthetic biomaterials, conversely, are more structurally manageable and have the potential to photocrosslink; however, they can also be more cytotoxic than natural materials, and capable of generating environments that may not be suitable for cell survival. Synthetic crosslinking agents, for example, can cause injury to cells, which can be avoided by employing natural crosslinkers.

In 3D printing, a variety of biomaterials have been described as bioinks. We can classify these biomaterials into natural and synthetic biomaterials, respectively. Over the past two decades, bioprinting has become an increasingly economically viable and accessible technology in tissue engineering research, biomedicine, and organ printing. However, application of bioprinting is uncommon, and the technology related to plant-related printing remains in its infancy. Several recent studies were...
conducted to generate new bioinks or bioink composites with improved cell viability, proliferation, permeability, and bioprintability to expand bioprinting techniques into the field of plant biology. Nevertheless, plant-based biomaterials and printing plant parts are slowly making advances in the area of bioprinting. Although, most plant cells are totipotent, that is, they have a strong potential to develop a tissue scaffold that acts as the precursor for an organ. From that organ, an entire plant can be generated, even if the environmental conditions are unfavorable. Despite cell totipotency, new plant cell-related discoveries in the field of bioprinting research are currently lacking. Moreover, the primary challenge to successful plant tissue printing is the lack of safe and intricate microarchitectures designed to mimic natural biological functions.

Plants have been extensively studied since the earliest times, and various essential chemical compounds with tremendous medicinal potential have been uncovered. Microorganisms, such as bacteria or viruses, can be countered with a well-functioning immune system. Therefore, stimulating the body’s natural defensive mechanism toward infections has been shown to be an effective technique. Alkaloids, polysaccharides, glycosides, terpenoid lactones, and flavonoids are important phytochemicals that have been linked to plant immunomodulation activities. These phytochemicals may serve as key compounds in the development of safe and effective immunomodulators for the treatment and prevention of viral illnesses. Natural products have been shown to influence the immune system in nonspecific ways. Plant extracts have been widely studied for their potential immunomodulatory characteristics in various regions of the world. For example, Acorus calamus rhizome extract inhibits the proliferation of various human and mouse cell lines. As a result, plants are frequently referred to as immunomodulators, which are chemicals that alter the immune system’s reaction to a threat. Immunomodulators modulate and strengthen the immune system, preparing it for any threat. Plant cells possess cell walls (CWs) while animal cells possess cell membranes, which allow them to be more flexible. Conversely, the CW is more useful for protecting the internal organ system.

Recently, the green bioprinting concept was established following the effective use of an extrusion-based bioprinting methodology developed for printing human cells for manufacturing photosynthetic microalgae-laden hydrogel scaffolds. Green bioprinting refers to printing plant cells or other plant-based fabrication. Primarily, green bioprinting is a promising emerging immobilization technology for plant cells that can allow the development of new bioprocesses and monitoring systems for secondary metabolite production.

In vitro synthesis from plant cell cultures could meet the growing demand for plant-derived components, such as secondary metabolites, in medicine, and cosmetic industries. However, further research is needed to improve process efficiency, which green bioprinting can potentially accelerate through the design of a specified immobilization matrix to enable the local and time-resolved analysis and modulation of substrate diffusion routes. The effects of cell immobilization on secondary metabolite production and extraction should thus be further investigated.

Secondary metabolites derived from plants are beneficial in pharmaceutical, food, and purely aesthetic additives. Immobilization can increase productivity by ensuring appropriate environmental conditions for cells; thus, various cell immobilization strategies have been used for plant cells, such as encapsulation in hydrogel beads (such as agarose, gelatin, alginate, and pectate), binding on the substrate surface of synthetic foams or fibers, and trapping hollow fibers in filter membranes. The use of the bioprinting approach, that is, cell encapsulation in hydrogels through computer-aided design based 3D printing, can advance the progress toward the success of a structured plant cell-laden 3D scaffold. The immobilization of plant cells in organized hydrogel matrices may allow the collection of spatially and temporally resolved data to analyze the impact of the immobilization matrix and culture conditions on secondary metabolite release. This would provide a better understanding of hydrogel-entrapped plant cell cultures. The fabrication of structured plant cell-laden hydrogel matrices by bioprinting could also enable the realization of architectures with defined macropore sizes and shapes that may be modified to induce specific conditions for plant cell cultures to produce large amounts of secondary metabolites. This is expected to increase productivity, make harvesting without cell loss easier, and allow the co-cultivation of cells derived from different species or even different taxonomic classifications. Furthermore, such structured immobilization matrices could be easily integrated into bioreactors (fixed-bed reactors), and twostage processes consisting of proliferative and production phases, which are promising for plant cells, could be easily established.

In addition, the present plant-based biomaterial production processes are inefficient and have unsustainable requirements. A new strategy for plant-based biomaterial production has been proposed by Beckwith et al., which significantly eliminates or reduces inefficiencies during pre-processing and agricultural stages. The feasibility of targeted culture strategies has been demonstrated using a Zinnia elegans model system. Gel-mediated cell cultures can be used to generate tunable plant-based constructs to mimic isolated plant tissues in pre-defined
structures. Isolated tissue-like generating materials require only small, non-sacrificial donations from the parent plant. Cells can then be collected, maintained, and expanded several folds from these biological starters to produce vast volumes of plant matter.

Compared to current procedures, the suggested isolated plant tissue-like production process promises numerous major benefits. For instance, reducing waste related to the biomaterial manufacturing process by producing only useful plant components (e.g., secondary xylem or wood) rather than undesirable or unusable plant parts (e.g., leaves, small twigs, roots, or bark). In vitro plant substrate growth can better control cellular composition, thereby improving the product’s material characteristics and chemical make-up tunability. Furthermore, with the controlled deposition of gel-scaffold material, the macroscopic substrate architecture may change to better fit application-specific needs, thereby allowing the creation of structures in a near-final form through casting or 3D bioprinting and minimizing waste further. In addition, novel methods or analysis can be easily replicated using a 3D printer, which is a key advantage of 3D printing in plant research.

This suggests that bioprinted plant constructs could be useful replacements for complex living systems. These constructs may aid in the resolution of unanswered biological questions, with potential for use as learning tools in plant research. Cellular components, such as the CW and mitochondria, are dynamic entities; hence, understanding these dynamics is essential to determine the morphophysiological response of cells to various growth conditions. This can be easily accomplished using 3D bioprinting, while the layer-by-layer printing method can be useful for studying subcellular and molecular dynamics within plant cells. Plant roots are highly capable of soil penetration as well as water and nutrient uptake; thus, a comprehensive understanding of plant roots could help with agricultural and environmental challenges such as the sustainability of soil resources. Plant physiological factors and external stimuli are considered in current studies on the expansion of below-ground root clusters, plant root elongation, and soil penetration. However, the root architecture and geometry, which are important for the movement of the root apex, have not been studied to analyze root penetration performance. Bioprinted root-based 3D models or biorobots may help clarify the geometrical and mechanical properties of root analogs. Decellularized spinach leaves, for example, can help explain leaf venation, while the chemical analysis of decellularized leaves can be useful for learning about the components present in plant leaves. Decellularization processes are used to create pre-vascularized, acellular tissue engineering scaffolds from a wide range of plant tissues. The rapid development of different plant species and their abundance provides several cost effective and long-lasting scaffold biomaterials, which could be used to form effective and sustainable bioinks.

Whole organ perfusion decellularization procedures can be modified for the synthesis of diverse plant tissues. For the preparation of spinach (Spinacia oleracea) leaf-based decellularized extracellular matrix (dECM) scaffolds, leaves are first cannulated through the petiole, while parsley (Petroselinum crispum) stems are cannulated through the basipetal end of the stem. A series of hexane treatments (98%, mixed isomers) and 1 × phosphate-buffered saline (PBS) solution are then used to remove the cuticles surrounding the leaf epithelial tissues on the plants.

The cannulas are perfused with 10% sodium dodecyl sulfate in deionized water for 5 days before perfusion with 0.1% Triton X-100 in a 10% sodium chloride bleach and deionized water solution for 48 h at a constant pressure of 152 mmHg. Cannulation and perfusion can also be used to decellularize the leaves of sweet wormwood (Artemisia annua) and hairy roots of peanuts (Arachis hypogaea) using the same solutions described above. After decellularization, tissues can be stored at 4°C in sterile deionized water for up to 2 weeks.

These dECM processes could help clarify the natural structural organization of higher plants, such as the transportation of nutrients through vascular tissues (xylem and phloem) from any part of the plant, such as the transfer of water from roots to the stem and leaves, and vice versa. Spinach leaves have been used as a model biomaterial for dECM-based natural scaffolds due to their availability, intricate vascular network pattern, density, and petiole with a large diameter, which simplifies the cannulation process.

The use of this type of process as a bioink preparation medium remains uncommon for plant printing but has attracted attention in the field of green bioprinting, as a result researchers are attempting to develop several methodologies to understand plant cellular dynamics, cell to cell translocation, leaf venation system, plant–environment interactions, and the plant immune system. New bioink processes for printing plant cells on a large scale in a simple and quick manner are also being developed.

The field of green bioprinting is vast with new discoveries being reported that require multiple studies to cover. Therefore, the primary goal of this review paper is to address various aspects of existing bioinks and their use in plant bioprinting.

2. Classification of bioinks

Bioinks are classified into two main categories: scaffold-based bioinks and scaffold-free bioinks. In bioprinting, scaffolds are fibrous, porous, or permeable three-dimensional (3D) biomaterials that allow biological liquids and gases to pass through while facilitating cell interaction, viability, and extracellular matrix (ECM) deposition with minimal inflammation and toxicity while biodegrading at a controlled rate.
2.1. Scaffold-based bioinks

Scaffold-based bioinks can combine cells into an exogenous support structure for biomaterials\cite{37-39} (Figure 2). dECM, microcarriers (spherical, porous structures that aid cell adhesion and growth), and hydrogels are common components of this supporting scaffold (dECM)\cite{37}. The scaffold further aids in the creation of functional tissue by promoting proliferation, differentiation, and cell growth while also providing biological and chemical cues, as well as mechanical strength\cite{40}. Scaffolds are supposed to degrade in time to form desired tissues while cells proliferate\cite{40}. Scaffold-based bioinks are often used because their degradation rate is similar to the rate at which cells construct the ECM\cite{41}. During the degradation process, scaffold biomaterials disintegrate, allowing living cells to occupy the new space and form a predesigned tissue structure\cite{37}. Controlling differentiation factors and rate of growth are optimized when the breakdown rate of the scaffold can be regulated\cite{41}.

Although scaffold-based bioinks are often highly biocompatible, they are sometimes prone to interruption of cell-to-cell interactions, material toxicity, slow degradation rates, undesirable immune reactions during \textit{in vivo} testing, and compromised mechanical properties due to the complete deterioration of the scaffold\cite{42-44}.

At present, scaffold-based bioinks are the most popular, due to their better innate structural properties, scalability, reproducibility, and affordability\cite{37,38,42}.

2.2. Scaffold-free bioinks

Scaffold-free bioinks are produced entirely using cells and their generated matrices, thus they do not require any additional biomaterials for support\cite{37,38,40}. Scaffold-free bioinks also refer to cells without the use of any exogenous biomaterial\cite{38}. Cell aggregation structures such as tissue strands, cell sheets, pellets, and spheroids make up these bioinks, which rely on the capacity of cells to self-assemble into bigger tissue structures\cite{37,38,41}. Scaffold-free bioinks waive the requirement for substantial cell growth due to tissue biomimicry, which improves cellular interactions, high seeding densities, and reduces immunological responses \textit{in vivo}\cite{40-42}. Using scaffold-free bioinks, living cells are printed in a manner that directly mimics normal embryonic growth\cite{3}. Clusters of the cells are deposited in a specific pattern to build larger, integrated, and functional tissue structures\cite{37}. Scaffold-free bioinks are promising in tissue fabrication since they are biocompatible, can facilitate ECM deposition with good cell-to-cell interaction, spread cells in a 3D environment, and enable the deposition of a high cell density\cite{45}. Bioink instigates

Figure 2. Diagrammatic illustration of scaffold-based and scaffold-free tissue engineering system. (from ref.\cite{47} licensed under Creative Commons Attribution license). The figure was created with BioRender.com.
rapid differentiation and accelerates tissue maturation, preserves cell functions and features for a long period of time, and provides a biomimicry microenvironment to increase ECM production, thus minimizing the time required for adaptation to an external environment and potentially overcoming rejection and tissue failure events that are common with scaffolds[46].

Therefore, scaffold-free bioinks provide high resolution and cell viability, carefully mimic the cell microenvironment of native organs and tissue for cell differentiation and proliferation, and preserve cell functionality and phenotypes for long periods of time[9].

3. Bioinks and tissue engineering

3D bioprinting allows the precise geometrical control of material deposition[48] and can automate, organize, and enhance the production of synthetic tissue. However, bioprinting tailored tissues with excellent print quality is not an easy task. The strict control over print accuracy and resolution in engineered organs and tissues can only become possible through a better grasp of bioprinting fundamentals and the incorporation of printing technologies[49,50]. Extrusion-based bioprinting creates uninterrupted cell-hydrogel extrudates while allowing heterogeneous material deposition, which is one of the three types of bioprinting methods. During hydrogel-based bioprinting, the strategic employment of support components in a support-assisted technique overcomes structural fidelity restrictions[50]. The employment of support materials in conjunction with building materials (e.g., bioink with cells) reduces the impact of gravity on the building material[48]. These characteristics are useful for simulating directional changes in cell alignment similar to those observed in native tissue on a macroscale.

Thus, with the development of novel bioinks, tissue engineering may expand. In the following sections, we discuss bioinks used in tissue engineering. Tissue engineering is an important area for potentially applying 3D printing; hence, several bioinks are being considered in this field.

3.1. Gelatin methacryloyl (GelMA) and alginate based bioink

In a 10 mM HEPES buffer, 10% w/v GelMA, and 2% w/v alginate were dissolved to develop this bioink. In ethanol, a photoinitiator comprising 10% w/v 2-hydroxy-4′-(2-hydroxyethoxy)-2 methylpropiophenone was dissolved, and 0.02% v/v of this solution was added to the construction material[48]. A total of $1 \times 10^6$ cells were placed into the bioink and extruded with a 25g needle; 30% w/v Pluronic F127 and 1 M calcium chloride were blended at a volume ratio of 3:1 for preparing the support material. Bioink (pressure, 1 bar; print speed, 700 mm min$^{-1}$) and Pluronic F127 (pressure, 3.5 bar; print speed, 600 mm min$^{-1}$) printing settings were optimized. Bioink was cured with a built-in UV pen that followed the print path at a rate of 10 mm min$^{-1}$. With the help of this bioink, a 0° – 90° grid was printed to analyze variations in cell alignment across various z-planes[49]. The cell culture was refilled 30 min after the support material was removed to ensure that the cellular behavior observed in the time point investigation of the bioprinted construct was attributable to cells embedded within the hydrogel. This prevents cells from dislodging from uncrosslinked materials and adhering to the surface of struts. The printed and seeded structures were cultured with cell media (high glucose DMEM, 15% FBS, 1% Penstrep) at 37°C and 5% carbon dioxide (CO$_2$). The cell culture media were replaced every 2 – 3 days[48].

These bioinks can be employed for macroscale cell alignment with support-assisted 3D bioprinting and coordinated tool path design (Figure 3).

3.2. Hydrogel fibers within GelMA bioink

Prendergast et al. have devised a new approach for hydrogel fibers with GelMA bioink that integrates synthetic fibers into bioinks aligned through biofabrication for direct cell alignment with the culture[51]. This was a synthetic microfiber (i.e., synthetically modified norbornene-functionalized HA [NorHA]) with regulated features (e.g., lengths) aligned through shear stress following the extrusion bioprinting of a cell-degradable bioink (i.e., GelMA) within agarose suspension baths[51]. GelMA was selected as a primary component of the ECM as it can be photocrosslinked to stabilize aligned fibers and degraded by cells during culturing to allow...
To produce NorHA microfibers, HA was first transformed to tetrabuta-lammonium (TBA) salt (HA-TBA) through a 2 h proton exchange reaction with a Dowex 50W proton exchange resin. The resin was removed through filtration, and the filtrate was neutralized with tetrabutylammonium hydroxide (TBA-OH) to a pH of ~7.02 – 7.05 before being frozen and lyophilized. HA-TBA was changed with norbornene-2-carboxylic acid (3 equivalent), 3-(dimethylaminopyridine (1.5 equivalent), and ditertbutyl decarbonate (0.4 equivalent) for 20 h at 45°C in the presence of nitrogen. The reaction was then quenched with water and diyalized against water for 7 days at room temperature using 0.25 g NaCl l¹ of deionized water (DI) before being lyophilized. Electrospun fiber mats were then created. Fiber mats were then cut into 1 mm² pieces and soaked in PBS for 30 min to produce microfibers. Mats were then sheared following hydration by continuously passing the solution through a needle. Then, 18 g (×40), 21 g (×40), and 23 g (×40) needles were used to shear the mats. The microfiber solution was first filtered using a 40 μm cell filter (BD, 352340), then through a 5 μm pluriStrainer after fragmentation. The residual solution was collected, centrifuged at 18,000 RCF, and kept at 4°C for up to 2 months in the dark.

GelMA was sterilized using a germicidal lamp under a laminar flow hood for 30 min before being dissolved in sterile solutions of photoinitiator (0.05 wt percent Lithium phenyl-2,4,6-trimethylbenzoylphosphinate [LAP]) and PBS for a final concentration of 5 wt%, unless otherwise stated, for bioink and suspension bath formulations. GelMA was dissolved by heating solutions to 37°C for 40 min. Unless otherwise noted, fibers were added at a concentration of 43 × 10⁹ mL⁻¹ and cells were introduced at a concentration of 5 × 10⁶ cell mL⁻¹. The formulation was placed in the printer using a 1 mL syringe. For agarose suspension baths, 0.5 wt% agarose was mixed with deionized water and autoclaved for 1 h on the magnetic stir plate and stirred at 700 rpm until it reached a temperature 25°C. This stock solution was then kept at 4°C for up to 3 months before use. Unless otherwise noted, the solution was diluted to 0.25 wt% with sterile PBS before bioprinting. The solution was then centrifuged at 500 × g for 5 min following dilution and before being added to the printing wells. Finally, the printing was initiated.

This adaptable multiscale biofabrication approach can be used to create 3D anisotropic fibrous microenvironments to engineer therapeutic connective tissues.

Future research must consider the effect of cellular alignment on cell matrix deposition and function, which is outside the scope of this review.

4. Biomaterials commonly used in 3D bioprinting

The development of bioink is one of the most difficult issues in the 3D bioprinting process. In general terms, the ink should indeed satisfy the physical, mechanical, and biological necessities of the printing process. To initiate, the ink must be biocompatible while also allowing cell proliferation and adhesion. Physically, the ink must be viscous enough to dispense from the printhead. Finally, the most important mechanical requirement is to provide sufficient strength and stiffness to ensure that the ink remains structurally intact after printing.

According to the biological necessities, bioinks can be categorized as natural bioinks, synthetic bioinks, and hybrid bioinks, which retain components of both natural and synthetic bioinks (Figure 4).

Natural biomaterials mimic the ECM structure or composition, biocompatibility, biodegradability, and self-assembling ability, making them ideal synthetic biomaterials. Synthetic biomaterials have their own advantages, such as photocrosslinking ability, stable pH, mechanical stability, and stable temperature responses; however, their poor cellular adhesion and biocompatibility, toxic byproducts, and mechanical property loss during degradation limit their applications. Overall, the combination of both natural biomaterials and synthetic biomaterials, that is, hybrid materials, is necessary to produce bioinks capable of mimicking both animal and plant tissues.

4.1. Natural biomaterials

Natural bioinks are polymers derived as biomaterial from naturally occurring resources. Several natural biomaterials are commonly used as bioinks in 3D printing, including agarose, alginate, cellulose, chitosan, collagen, dECM, and silk.

4.1.1. Agarose

Agarose is a natural polysaccharide obtained from red seaweed that comprises repeating  β-D-galactose and 3,6-anhydro-α-L-galactose disaccharide units (Figure 5). As a member of the carbohydrate family, agarose has a thermo-reversible gelling mechanism and high biocompatibility, thus agarose is frequently used in tissue engineering applications.
4.1.2. Alginate

Alginate is a brown algae-derived anionic polymer composed mainly of blocked copolymers, with α-(1-4)-L-guluronate and (1,4)-linked β-D-mannuronate residues arranged in different sequences and ratios depending on the alginate source\(^69,70\). Alginates are widely used in biomedical applications such as wound healing, drug delivery, and tissue engineering because of their low cost and biocompatibility\(^17,69\). The ability of alginate to make hydrogels with characteristics analog to the ECM of tissues is the main advantage of alginate-based bioinks\(^70\) (Figure 6).

4.1.3. Cellulose

Cellulose, a very rigid polysaccharide made up of (1 – 4) linked β-D-glucopyranosyl units linked together, plays are the primary structural substance in plant cells\(^73,74\). Carboxymethyl cellulose is a water-soluble form of cellulose ether that is often used to change the density of other polymers with subpar rheological qualities\(^75\). When cellulose chains are well organized, they form composed cellulose nanocrystals (CNC) which can improve shear-thinning behavior and enhance mechanical strength\(^76\). Cellulose, as well as CNCs, are often used in bioinks because of its ability to improve the porosity of the structures, its elasticity, its ability to bind with other substances, and enhancement of bioink viscosity\(^77,78\). In addition to their biocompatibility, CNCs possess antibacterial properties, making it an appealing option for the applications in wound dressing\(^79\) (Figure 7).
4.1.4. Chitosan

Chitosan is a polysaccharide that forms naturally when chitin is deacetylated\cite{80,81}. Chitosan is normally insoluble in water, although it can be dissolved in solutions with pH 6.2 or lower solutions. Chitosan is also biodegradable, bio adhesive, non-toxic, renewable, and biocompatible; however, it also has low mechanical strength, which limits its use in the production of hard tissues such as cartilage\cite{80,81} (Figure 8).

4.1.5. Collagen

Collagens are the most widespread proteins found in mammals, accounting for roughly 30% of the average total protein mass in mammals\cite{83}. Collagens are hydrophilic proteins that play significant structure roles in the ECMs of cells\cite{34,83} (Figure 9). Collagens feature triple helical domains composed of three polypeptide chains. Collagens come in 28 different varieties, each with a unique number of triple helices and chain combinations\cite{34,83,85}. Collagens possess integrin-binding domains that enhance proliferation, cell adhesion, and attachment, and they are immunologically inactive, meaning they do not exhibit immune responses\cite{37}.

4.1.6. dECM

Decellularization of tissues by several physical and chemical processes such as detergents, enzymatic agents, freeze-thaw cycles results in the formation of dECMs\cite{85}. The purpose of tissue decellularization is to eliminate tissue’s cellular components while maximally conserving the structure and substance of the ECM\cite{15} (Figure 10). The retention of the properties of the ECM has several advantages with respect to use as a bioink component, and as a means of removing crosslinkers\cite{85}.

4.1.7. Silk

Silk fibroin (SF) is a highly versatile natural protein derived from silkworms\cite{3,87,88}. In tissue engineering, SF is quite valuable because of its mechanical characteristics, biocompatibility, and easily regulated degradability. Shear thinning qualities also make this natural fibrous polymer perfect for extrusion bioprinting\cite{89}. SF is an appealing natural hydrogel component because it can be physically crosslinked, eliminating the requirement for harsh crosslinking chemicals, and it can allow chemical interactions beyond simple covalent interactions\cite{10} (Figure 11).

There are a number of other biomaterials used in the bioprinting process, such as fibrin, dextran, gelatin, HA, Matrigel, Gellan gum, and more; however, the seven biomaterials discussed above are the most commonly used\cite{15}. A summary table is provided below to highlight additional information about these biomaterials (Table 2).

4.2. Synthetic biomaterials

Natural polymers or hydrogels are able to directly mimic the native ECM and providing the appropriate micro-environment for cell adhesion and proliferation with limited adjustable capabilities\cite{56}. As a result, these natural polymers are merged with synthetic polymers or other natural polymers to form more stable, customizable 3D bioprinting structures\cite{3}. Unlike natural polymers, synthetic polymers neither facilitate nor promote cellular adhesion. Because of this ability, they are promising options for modifying features including mechanical qualities, printability, and cross-linking, and so on. Pluronic and poly(ethylene glycol) (PEG) are the most often utilized synthetic polymers within the field of 3D bioprinting\cite{100}. Polyethylene (glycol) diacrylate (PEGDA)
and poly (ethylene glycol) methacrylate/dimethacrylate are the most prevalent PEG hydrogels utilized for bioink materials[61]. The transitioning temperature of these hydrogels varies; poloxamer is an aqueous, polar, and non-polar organic solvent soluble copolymer[106], while pluronic F127 poloxamer is most commonly used for 3D printing. At 4 – 5°C, this material is liquid, and at temperatures >16°C, it becomes a gel. In water, poly (2-hydroxyethyl methacrylate)-PHEMA forms a clear hydrogel; hence, it is suitable for bio scaffolds as it allows oxygen (O$_2$) to diffuse through the layer[107]. Poly (L-lactic acid) and poly (D, L-lactic acid) can be dissolved in dioxane and combined with bone morphogenic protein ground into particles and suspended in deionized water to create bone scaffold material[108].

5. Application of bioinks for printing algal cells

The ability to fabricate algal and microalgal cells is one of the most promising advances in the field of 3D bioprinting[25]. Algae can absorb solar energy and convert carbon dioxide into usable products, including compounds that are used in food, biofuel, cosmetics, and pharmaceutical products, including substances with anti-inflammatory, antibacterial, and/or anti-tumor properties[109]. Biofiltration or the extraction of heavy metals, nutrients, and industrial pollutants from wastewater, is another key application for photoautotrophic microalgae[110,111]. Furthermore, because microalgae are sensitive to a wide range of pollutants, they have been used to develop biosensors for assessing the quality of the aquatic environment[112]. To date, bioinks composed of natural, cell-friendly biopolymers, including alginate, starch, silk, and carrageenan, have been used to bioprint microalgae [25,113,114]; however, there is still potential for utilizing synthetic biopolymers as bioinks for printing algal cells.

![Figure 8. Extraction and application procedure of chitosan (from ref. [62] licensed under Creative Commons Attribution License).](image)

![Figure 9. Diagrammatic representation of the printing process using collagen-based bioinks. Reprinted with permission from ACS Appl. Mater. Interfaces 2021, 13, 6, 7037 – 7050. Copyright 2021 American Chemical Society[84].](image)
5.1. Natural bioinks

5.1.1. Environmentally sustainable microalgal silk structures

For the preparation of bioink, silk is chosen for its safe, strong mechanical qualities and compatible use, environmentally friendly source, and ability to make a variety of materials and through several experiments, it had been proven that the gelation and mechanical kinetics of the hydrogel components are specifically designed for printing\(^\text{[115]}\).

Based on biocompatibility and gelation conditions, living microalgae can be hosted in silk protein-based hydrogel compounds using this reference for the preparation of this bioink silk hydrogel constructs contain a strain of marine algae in the genus *Platymonas*.

Previous research on this form of silk hydrogel found that these algae-based, silk hydrogel structures supported cell proliferation for at least 4 weeks, as well as continuous photosynthetic activity for over 90 days\(^\text{[114]}\).

Microalgae within this ink were concentrated at 1,200 rpm for 5 min, and then harvested to yield microalgae or microalgae-based silk inks used in the fabrication of silk hydrogels (Figure 12). The enriched microalgal solution was then mixed within a SF solution (26 % w/v) in a 2:3 ratio per volume, yielding a silk concentration of 15.6 % w/v. Microalgal media was only used for ink formulations that did not already contain microalgae. To improve the viscosity of the ink mixture, which is more conducive to printing, the microalgae or microalga-based silk mixtures were blended with a 15% w/v hydroxypropyl methylcellulose.
| Biomaterials | Derived from | Compositions | Advantages | Disadvantages | Reference |
|-------------|--------------|--------------|------------|--------------|-----------|
| Alginate    | Brown algae  | Alginate is a group of linear copolymers made up of blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues. | i. Can be used for bioprinting at various concentrations (2–4%).
ii. Construct stability
iii. Biocompatible, low toxicity, and affordable
iv. Crosslinking is simple. | i. Cellular responses differ depending on the source of stimulus.
ii. Because of its very hydrophilic nature, it has low cell adhesion and protein adsorption.
iii. *In vitro* degradation is rapid (40% within 9 days), and additional dopants are required for stability.
iv. Absent cell-binding motifs | [92] |
| Agarose     | commonly extracted from red seaweed (red algae) species such as Gracilaria, Ceramium, Gelidium, Pterocladiad, Acanthopeltis, and Campylaephora | Made of repeating units of agarobiose, which is a disaccharide unit made up of (1,3) linked β-D-galactopyranose and (1,4) linked α-3,6-anhydro-L-galactopyranose | i. Shape fidelity is maintained over a wide temperature range.
ii. Soft tissue stiffness in the natural range
iii. Biodegradable
iv. Non-toxic
v. Cost effective | i. Cell adhesion and protein adsorption resistance
ii. Can hybridize with another polymer to provide biological characteristics
iii. Dispensing requires a high temperature (70°C) and gels at low temperatures. | [92,93] |
| Cellulose   | a biopolymer found in abundance in plant cell walls and released in pure form by various microorganisms. | It is mainly composed of repeated units of b-D-glucopyranose that are held together by covalent bonds between the OH groups of the C4 and C1 carbon atoms. | i. Capacity to improve the structure’s porosity.
ii. Elasticity
iii. Binding ability to other substances
iv. High viscosity of bioinks
v. Biocompatible
vi. Biodegradable | i. Inadequate thermal stability
ii. Moisture absorption
iii. Incompatible with hydrophobic polymers | [94,95] |
| Chitosan    | Chitin is found in nature as crystalline microfibrils that form the structural parts of arthropod exoskeletons | Chitin is a polymer of N-acetyl-D-glucosamine, and when deacetylated, the repeating units in the polymer are primarily devoid of the acetyl functional group, i.e., as β-1,4-D-glucosamine, and | i. Biocompatible
ii. Biodegradable
iii. Non-toxic
iv. Antimicrobial
v. Hydrating agent
vi. Bio-adhesive
vii. Renewable | i. Low mechanical strength
ii. Poor gelation | [96,97] |

(Contd...)
Table 2. (Continued).

| Biomaterials       | Derived from                                                                 | Compositions                                                                                     | Advantages                                                                                     | Disadvantages                                                                                     | Reference |
|--------------------|------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|-----------|
| Chitin             | Derived from cell walls. Chitin is also created by a variety of other living organisms in the lower plant and animal kingdoms for its strong reinforcing and strength protection qualities. Chitosan is generated from deacetylated chitin. | the polymer is known as chitosan.                                                               | i. Excellent printability                                                                      | i. Due to shrinkage, inconsistent deformation structures limit its application for high-resolution 3D printing. | [92,98-100] |
| Collagen           | Collagen is the primary structural protein found in the connective tissues of animals, typically found in fibrous tissues including tendons and ligaments, as well as bones, cartilage, cornea, intervertebral discs, stomach, and blood vessels. | Most collagens have two α-1 chains and one α-2 chain. A single chain consists of a left-handed helix with 3.3 residues per turn. To construct a right-handed superhelical structure, the α-chains are twisted together. Between the residues of various chains, hydrogen bonds are present. Both collagen chains have a three-amino-acid repeating unit, with glycine (Gly) accounting for one-third of all amino acids in each chain. Proline (Pro) and hydroxyproline (Hyp) regularly follow each other, with the sequence Gly-Pro-Hyp making up approximately 10% of the molecule. | i. Low immunogenicity                                                                              | ii. Sterilization causes structural changes. iii. Mechanically weak (elastic moduli of 1 kPa), hence cannot construct self-supporting scaffolds. | [92,101-103] |
| Decellularized     | Diverse organs and tissues have been decellularized for producing natural ECM using several methodologies involving, enzymatic, chemical, and                                                                 | Collagen and proteoglycans, two key components of ECM, are secreted by cells and organized in a manner particular to individual tissue types. It also contains a growth factor and cytokine reservoir. | i. Creates a microenvironment of natural extracellular matrix ii. Directs a tissue-specific lineage and maintains the phenotype of mature differentiated cells. | i. Detergent-induced protein denaturation ii. Low mechanical qualities necessitate a hybridization with a polymeric matrix to provide stability, which must be removed subsequently. | [92,101-103] |
| Biomaterials | Derived from | Compositions | Advantages | Disadvantages | Reference |
|-------------|--------------|--------------|------------|---------------|-----------|
| **Silk**    | There are many different forms of silk fibroin in nature, yet those generated from silkworm and spider silk are the most studied. Due to its widespread availability, silkworm silk *Bombyx mori* is unquestionably the better known of the two major sources. | The fibroin chain is made up of amino- (N-) (130 amino acids) and carboxyl- (C-) (100 amino acids) peptide domains; whereas the C-terminus is conserved in both silkworms and spiders, the N-terminus is conserved only in spiders, and the sequence in silkworms varies greatly. | i. Mechanism of biomineralization ii. Structural modification is simple. iii. under control environment iv. Possibility of introducing physical crosslinks (−sheets) to cause sol-to-gel transitions without using solvents, heat, or UV light. v. High cellular viability for at least 1 month vi. Adaptable structural modification | i. The fact that decellularization causes non-reversible secondary structural changes in collagen is commonly overlooked by researchers. ii. As the amino acid sequences of fibroins are diverse among different species, either due to environmental impacts or evolution, batch-to-batch variance exists. iii. The rheology of the bioink must be optimized. | [92] |
| **Gelatin** | Gelatin is mainly derived from mammalian cells. The primary sources of gelatin are collagens from pigs, cows, Sometimes, gelatine can also be extracted from the skin and bones of various kinds of fish. | Gelatin is composed of 18 different types of complex amino acids, the most important of which are glycine, hydroxyproline, proline and with the remainder consist of other well-known amino acid families such as glutamic acid, alanine, arginine, and aspartic acid. Gelatin’s chemical structure also includes α-chains (one polymer/single chain), β-chains (two covalently crosslinked α-chains), and γ-chains (three covalently crosslinked α-chains) with molar masses of approximately 90×10³, 180×10³, and 300×10³ g/mol, respectively. | i. Phase transition behavior in a thermo responsive system ii. Encourages cellular proliferation and the production of ECM. iii. Low immunogenicity. | i. Mechanically weak at physiological temperatures ii. Stability of printed constructions necessitates the incorporation of polymeric dopants or crosslinking. | [92,104] |
(HPMC) solution at various weight ratios (3:7, 4:6, 5:5, and 6:4), hereafter referred to as silk to HPMC ratios. Three doses of horseradish peroxidase (HRP) were then added to the ink mixture at volumes of 60, 120, and 180 units/mL\(^{[114]}\).

Then, 3D models were first designed with 3Ds MAX and printed using the synthesized algae-based silk hydrogel structures (Figure 12). For printing, the ink was extruded into a medium containing 0.01% w/w hydrogen peroxide (H\(_2\)O\(_2\)), which is generally used to make ink without microalgae while deionized water is used and for making ink with microalgae, to start cross-linking immediately after printing. The concentration of H\(_2\)O\(_2\) in the solution was set to facilitate efficient gel cross-linking while ensuring maximal cell survival. When the microalgal silk hydrogel had sufficiently cross-linked, the H\(_2\)O\(_2\) solution was replaced with microalgal media to facilitate cell proliferation\(^{[114]}\).

The long-term cell survival and performance of these systems allow them to be employed for a variety of purposes, including O\(_2\) replenishment and carbon dioxide reduction with the goal of a greener, healthier indoor environment\(^{[114]}\).

This strategy was successfully used to host microalgae, producing a microalgal silk bioink with mechanical properties and gelation kinetics suitable for 3D printing. The silk hydrogels offered a supportive environment for the long-term growth and photosynthetic activity of encapsulated microalgae. The proliferation of microalgae was observed for more than 4 weeks, with a sustained photosynthetic activity for at least 90 days. This stability, long-term functionality, and printability of the support material poses potential environmental benefits\(^{[114]}\).

5.2.2. Post-printing determination of algal cell stability

Alginate, a methylcellulose (mc) hydrogel-based bioink, was chosen for this experiment\(^{[116]}\). For algal cell, *Chlamydomonas reinhardtii* strain cc125 was chosen.

Alginate is an algae-derived natural polymer, whereas mc is a polymer made up of several linked glucose molecules. First, 3 – 4 g of algicnic acid sodium salt was added to 100 mL of deionized water. On a heated plate with a magnetic stirrer, the alginate solution was swirled at 900 rpm for 5 h. Once the alginate was heated to 90°C, 6 g of mc powder was added, and then the solution was sterilized in an autoclave for 1 min at 121°C. Algal cells derived from TAP-algae (Tris-acetate-phosphate algal solution) were introduced to the alginate, followed by a mc solution, then the solution was cooled until it reached room temperature\(^{[116]}\). The concentration of algal cells in the prepared bioink was 150,000 cells/mL.

After the preparation of the bioink, the printing process began (Figure 13). After printing, the samples were immersed in 100 mM calcium chloride (CaCl\(_2\)) solution for 4 min for crosslinking\(^{[117]}\). Cells in printed structures are immobilized, and algal cells as those used here can grow indefinitely in this state\(^{[27]}\). As a result, bound algal cells can multiply more rapidly, creating a higher cell density, and generate more metabolites per cell than cells dispersed in a liquid medium or less viscous bioink\(^{[28]}\).

The effects of extrusion pressure and needle diameter on the number of algae cells in printed samples were explored in this study, leading to the following findings:

1. A higher extrusion pressure reduced the number of algae cells in printed samples; this pattern was observed 3 and 6 days after printing.

2 For TAP algal cell preparation, 100 ml of tris-acetate-phosphate (TAP) culture media was prepared in a 150 ml flask. Cells of the frequently used *C. reinhardtii* algae strain cc125 were streaked from a petri dish and added to the flask. To maintain sterility, the addition was performed in a biosafety cabinet. The flask containing the TAP-algae solution was shaken for 72 h. The shaker was set to 100 rpm and kept at 22°C under light bulbs to allow the algae cells to grow. Thereafter, in a fresh flask containing 100 ml of liquid TAP medium, 10 ml of the TAP-algae solution was added, yielding a 110 ml TAP-algae solution. The TAP-algae solution was then transferred into a new flask and exposed to light for 24 h to allow algal cell proliferation.

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**Figure 12.** Silk/HPMC ink concrete mixtures in 3D printing. (A) A diagram depicting the printing method of silk/HPMC ink combination with microalgae. (B) 3D printed constructions (a square-based pyramid and a bar spanning two conical-shaped pillars) with silk to HPMC ratio of 6:4 and a 180 unit/mL HRP ink mixture. The 3ds MAX designs are seen in the insets. Scale bar: 1 cm. Reprinted with permission from ACS Biomater. Sci. Eng. 2019, 5, 9, 4808–4816. Copyright 2019 American Chemical Society\(^{[114]}\).

**Figure 13.** Bioink preparation procedure.
ii. As needle diameter increased, so did the number of algae cells in printed samples; this pattern was observed 3 and 6 days after printing.

5.2.3. Photosynthetic living components

The hydrogel was developed by encapsulating an alginate hydrogel matrix onto non-living bacterial cellulose for 3D bioprinting of photosynthetically active microalgae (C. reinhardtii) capable of generating energy and O$_2$\cite{118}. The calcium alginate-based hydrogel enhances nutrient permeability, light transmission, and gas (O$_2$ and CO$_2$) emission, all of which are important for microalgal growth\cite{119}. Bacterial cellulose, in general, is a flexible, cell-friendly, and durable biopolymer with exceptional properties such as toughness (2 – 25 MJ m$^{-3}$) and tensile strength (73 – 194 MPa)\cite{120,121}. The fermentation of bacteria such as Komagataeibacter rhaeticus and Gluconacetobacter hansenii produces bacterial cellulose\cite{122,123}. Bacterial cellulose possesses a nano-fibrous architecture and absorbent properties that, if further employed as a substrate for microalgal bioprint, could allow nutrients to circulate and penetrate into microalgal cells, thus promoting the growth of algae\cite{124}. In addition, these hydrogels are perfectly biodegradable and biocompatible, and are good encapsulators as they do not interfere with cell–cell interactions while still allowing the transport of water\cite{61}. This hydrogel can be utilized to fabricate living microalgal materials as well as for the development of low-cost microalgal bioprinters\cite{118}. Microalgae have been printed onto agar and bacterial cellulose substrates using home-built bioprinters (Figure 14). Microalgae can be bioprinted into various sizes and pre-defined geometries as mono- or multi-layered constructs using this technology. Surprisingly, the bioprints may be removed from the bacterial cellulose and reattached to new bacterial cellulose surfaces while maintaining adhesion. The resistance of these bioprinted microalgal structures to physical deformation and immersion in water demonstrates their physical stability. The bioprinted microalgal cells retain good viability for at least a month. The patterned microalgae in bioprints can also be regenerated to make new bioinks. After removal from the nutrient broth, the microalgal cells bioprinted onto bacterial cellulose could live for at least 3 days, with their longevity being extended further when placed onto fresh agar. Overall, these regenerative photosynthetic living materials composed of microalgae bioprinted on bacterial cellulose have a wide range of potential uses, including adhesive labels, photosynthetic bio-garments, and artificial leaves. Finally, living materials can be 3D printed by depositing living cells (bioink) layer by layer onto a non-living matrix (substrate). The composition of the bioink and the nature of the printing substrate both play important roles in preserving the viability and functionality of cells in the resulting bioprinted materials, as well as the overall 3D structure. When a bioink composed of sodium alginate and microalgae is printed onto a substrate composed of bacterial cellulose and calcium chloride, an alginate hydrogel can form wherein microalgal cells are immobilized.

The bacterial cellulose is placed on top of a microalgal nutrient medium (minimal medium or carbon-supplemented medium) to ensure that microalgae can grow inside the bioprints on the bacterial cellulose. The bacterial cellulose supporting the living bioprinted microalgae can then be peeled off and used in different ways. Microalgal cells in bioprints can also be regenerated and used as new bioinks in subsequent bioprinting processes.

5.2.4. Hydrogel filters for copper removal

The bioink in this study was prepared with sodium alginate containing the C. reinhardtii algae strain cc125\cite{125}.

![Figure 14. Regenerative bioprinting of photosynthetic living components (from ref.\cite{118} licensed under Creative Commons Attribution license).](image)

![Figure 15. (A) Design of the filter; (B) Custom filtration setup developed for the experiment (from ref.\cite{125} licensed under Creative Commons Attribution license).](image)
To produce the bioink, sodium alginate was first dissolved in distilled water at a concentration of 4% (w/v)\(^{[126]}\). Using a 25 mL hypodermic syringe and a #21 needle, the polymer solution was dropped into the gelation media comprising a 250 mL CaCl\(_2\) solution at a defined concentration (w/v) under continuous stirring at room temperature. The beads were then cured in the gelation medium for 15 min, removed and washed with distilled water, and then dried at 30°C in a debris room. Beads crosslinked with BaCl\(_2\) and AlCl\(_3\) were made in the same way. Notably, the drying process affects the stability of beads. The porosity of the beads decreases when they partially dry. The complete dehydration of the beads can cause surface cracking, which can make the beads more prone to surface erosion when rehydrated. The swelling and deterioration indices are thus likely to be affected.

Furthermore, water in hydrogel exists in two states: bound water and free water. As a result, it is possible that when the beads were dried at 30°C, free water may escape while the bound water remains; hence, crosslinking ions may be present when the beads are hydrated. The distance between the syringe and the gelation medium, the number of drops of polymer solution falling into the gelation media per minute, and the temperature were all kept consistent throughout the experiment.

Algal cells derived from TAP-algae were then introduced into the hydrogel. The TAP-algae solution was prepared\(^{[115]}\). Fresh liquid TAP media was used for the algal cells during the preparation of the TAP-algae solution. Since it included fresh nutrients rather than accumulated cellular waste elements like methane and free radicals, the fresh medium was perfect for cell development\(^{[127]}\). Following the preparation of algal cells, cells were transferred into the previously prepared hydrogel. The hydrogel bioink (used to print algae-containing hydrogel filters) had a cell concentration of 150,000 cells mL\(^{-1}\). This prepared hydrogel was used to print the filters on a 10 cm Petri dish (Figure 16). The filters were in the shape of square disks with a length of 25 mm and a thickness of 1.5 mm and included 8 holes with a diameter of 2 mm each. The printing speed was 6 mm s\(^{-1}\), while the extrusion pressure was 95 psi to deposit layers with a thickness of 0.1 mm. After printing, 5 mm of 4% w/v CaCl\(_2\) solution was added to the petri dish holding the printed filter for 2 min to allow alginate crosslinking\(^{[127]}\). Crosslinking generates chemical linkages between the polymer chains of the printed filters, allowing the retention of their printed shape (Figure 15). After crosslinking was completed, liquid TAP media were poured over the filters to allow algal cell proliferation. The algal cells containing the filters were placed at room temperature for 4 days below light bulbs once they had proliferated. This is the 1\(^{\text{st}}\) time that the 3D printing of algae-infused hydrogel filters has been utilized to remove copper from contaminated water\(^{[125]}\).

## 5.2. Hybrid bioink

### 5.2.1. Textile biomposites with photosynthetic properties

Single-cell photosynthetic microalgae, which absorb CO\(_2\) and release O\(_2\) as a byproduct, have been utilized in recent years. They have their own set of requirements like living organisms, including the need for an appropriate pH, light, temperature, and nutrient supply\(^{[128]}\). The construction and fabrication procedures for these unique “living organisms” could be reevaluated. For example, biogel printing has become popular in the area of material research, with potential applications in food industries and pharmaceuticals, wherein experiments are ongoing to optimize the assimilation of living cells into digital printing methods\(^{[129,130]}\).

Kappa-carrageenan, chitosan, Aloe Vera, and a clay-based (Auro clay) binder have all been used as biogel matrices for printing (Table 3).

To prepare this type of ink cell, the eukaryotic green microalga *Chlorella vulgaris* (paraphylum Chlorophyta) is often used in conjunction with the selected microalga because it is considerably more resilient than other microalgae and cyanobacteria. In addition, these cells are compatible with a variety of environments, including office and large public buildings, which provide a uniform high-intensity light cycle, vast surface area, and constant temperatures.

To create the foundation of the bio-gel matrix, at room temperature, kappa-carrageenan (from >99.9% pure powder) has been added to a dilution series of BG11...
The solution was then agitated until a consistent viscosity is reached. After achieving a gel-like consistency, *C. vulgaris* was added to the solution, which is then mixed until it was completely homogenous, after which *Aloe Vera* was added. For chitosan treatments, chitosan powder (food grade) has been dissolved in a dilution series of BG11 after the addition of acetic acid (0.3 mL/10 mL) to the solution. Once the final solution is gel-like in consistency and all components are homogenous, printing can begin.

The potential of *C. vulgaris*, which can grow in a low-moisture atmosphere, can be further explored in bioinks, as it can survive in numerous matrices and on a diverse range of different textiles, allowing for a broad array of possible implementations in multiple fields. This work demonstrated that *C. vulgaris* can be grown in a low-moisture environment in a variety of matrices and on a variety of textiles, allowing for a broad spectrum of applications within the fabric of the building. The original study aims were to create a novel type of biological material fabricated using digital 3D printing methods and investigate the progression of this material in terms of cell development and migration, thereby demonstrating live behavior not typically associated with materials used in the construction industry. The findings suggested that living textile materials would require a certain maintenance schedule to prevent sudden moisture evaporation, which could result in cell death and matrix flaking.

### 3D printing of photosynthetic gels

This gel was made using a combination of Jeffamine polyglycol amines and PEG diglycidyl ether in water, poly (ethylene glycol) diacrylates with various curing agents, and calcium-crosslinked alginate, among other gel compositions.

In this preparation, *Chlorella* sp. (a single-celled algae genus that is green and photosynthetic) was used as the algae. *Chlorella* in suspension was obtained and grown in dishes on wet bentonite clay under a 12 h/day “grow light” fluorescent bulb. Since a layer of algae can be scraped off from the clay and incorporated into the gel for printing, this approach is convenient.

Approximately 5% of the algal clay paste and 7% of fumed silica (Cabosil was used to provide solution rheology with a consistency similar to that of toothpaste) were combined into the gel for printing. On a modified Taig CNC milling machine controlled by Mach3 software, this paste was placed in syringes and extruded by a stepper-motor powered actuator as it was moved to construct porous 3D “logpile” structures. The alginates were formed after the pastes and were sprayed with a 100

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3 Bradykinase reduces inflammation when applied topically.

| Material                  | Material Information                                                                 | Reference |
|---------------------------|-------------------------------------------------------------------------------------|-----------|
| Kappa-carrageenan         | The sulfated polysaccharide Kappa-carrageenan is found in red seaweed and often used as a food additive. Carrageenan is a carbohydrate that can thicken and stabilize foods and is used as a fat substitute in some goods. It forms a gel when extracted from seaweed and can be used to improve food texture. | [132]     |
| Chitosan                  | A linear polysaccharide developed by treating the chitin shells of shrimp and other crustaceans with an alkaline material, such as sodium hydroxide, to produce randomly dispersed β-linked D-glucosamine and N-acetyl-D-glucosamine. | [133]     |
| Aloe vera gel             | *Aloe barbadensis* miller is a xerophytic, perennial, shrubby, or arborescent, succulent, pea-green hue plant that belongs to the Asphodelaceae (Liliaceae) family. *Aloe vera* contains 75 potentially active constituents, including vitamins (e.g., A, C, and E), enzymes (e.g., aliase, alkaline phosphatase, amylase, bradykinase, carboxypeptidase, catalase, cellulase, lipase, and peroxidase), minerals (e.g., calcium, chromium, copper, selenium, magnesium, manganese, potassium, sodium, and zinc), sugars, lignin, saponin, salicylic acids, and amino acids. | [131,134] |
| Auro clay paint           | AURO High-grade Clay Paint is the world’s first ready-to-use preservative-free clay paint. The composition comprises clay, water, cellulose, mineral fillers, titanium dioxide, Replebin®, mineral pigments, silicates, and potassium. Castor and rapeseed oils are commonly used to produce these types of surfactants. | [131]     |
mM calcium chloride solution. Calcium crosslinking was used to crosslink the alginate gels, which were then kept in 10 mM calcium chloride solutions. These gels gradually turned dark green when submerged in water and lit. To show photosynthetic activity, the undamaged “logpiles” were gently agitated in a flask of water and the dissolved O₂ concentration of the water was measured. The O₂ level climbed above that of water saturated with air during the lighted period, and then fell as the light was turned off overnight. The alginate logpiles broke up over several weeks, either due to bacterial action or simply because the algae consumed the alginate. In some samples, white fungal or bacterial proliferation was visible[135].

Photosynthetic algae often create antimicrobial compounds to defend their colonies against infection by other species, as well as symbiotic bacteria that help the algae to reproduce.

Cell cultures are heavily reliant on specific formulations that offer the nutrients required for cell multiplication. This work shows that photosynthetic algae can be printed and grown in biopolymer gels, although it was stated that a synthetic gel may be a better substitute for the long-term study of stable “tissues.” As cells can die while their chlorophyll stays intact, there was no evidence that the cells in the synthetic gels were alive in the dark. Chlorophyll can photodegrade, hence the presence of the green color under light for an extended period of time indicates that the cells were alive and produced chlorophyll, even when they were not multiplying[135]. The cells multiplication in the alginate gels can be observed by the change in color from light to dark green.

As synthetic gels are denser than alginites, the difficulty in proliferation can possibly be attributed to the toxicity of one of the components or diffusional limits. Plant cells are frequently provided with glucose when cultivated in the laboratory, making photosynthesis unnecessary as an energy source.

For growth, cells also require a source of nitrogen and phosphorus, as well as numerous minerals.

Different cells could also be combined into a gelling matrix and shaped into porous structures utilizing 3D printing.

A summary of above discussed bioinks is provided in Table 4.

6. Application of bioinks within the field of plant bioprinting

6.1. Natural bioink

6.1.1. Green bioprinting: Fabrication of plant cells

A hydrogel blend (alg/aga/mc) containing alginate (alg, 2.8 wt%), agarose (aga, 0.9 wt%), and mc was developed for bioprinting with plant cells (3 wt%)[27]. At a temperature of 120°C, alginic acid sodium salt, agarose, and sucrose (3 wt%; as nutrients) were dissolved in deionized water. This mixture and mc powder were autoclaved individually for 20 min at 121°C. After allowing the mc to cool to room temperature, the mixture was added to the alginate/agarose blend (alg/aga), stirred to form a homogeneous plotting paste, and incubated for 2 h to allow the mc to swell. At room temperature, the rheological properties of alg/aga and alg/aga/mc were measured using a rotary rheometer with a plate/plate measurement device (30 mm, h = 0.1 mm). The viscosity was measured for 300 s at a constant shear rate of 10 s⁻¹. Shear thinning experiments were carried out by increasing the shear rate from 0 to 50 s⁻¹ over a period of 600 s (increments of 0.08 s⁻¹) and corresponding viscosity was determined. Every experiment was carried out in triplicates.

For plant cell bioprinting, an in vitro cell culture of basil (Ocimum basilicum L. var. purpurascens Benth. “Cinnamon Basil”) was used. The initial callus culture was obtained by transforming a sterile shoot culture with Agrobacterium tumefaciens C58 (wild type). From the obtained callus, a basil suspension culture was established by transferring 2 g of biomass (fresh weight) into 50 mL MS medium with an initial sucrose content of 3 wt% and a pH value adjusted to 5.7 ± 0.1 before sterilization. The basil suspension culture was kept in 50 mL MS medium (250 mL Erlenmeyer flasks) with weekly passaging by transferring 20 vol% of the cell culture to 80 vol% fresh MS medium. All cultivation in this study was carried out in the dark on an orbital rotary shaker (110 rpm, 20 mm shaking diameter) at room temperature. Plant cells were collected from the MS medium on day 7 after passaging using a glass frit filter (pore size 160 – 250 μm) for bioprinting. One gram of concentrated biomass was gently mixed into 10 mL of prepared hydrogel paste. The cell-laden hydrogel was then printed, and the resulted scaffolds were crosslinked.

Bioscaffolder 3.1 was used for extrusion-based 3D plotting. The hydrogel pastes with the plant cells were discharged using a conic dosing needle with an internal diameter of 610 μm, plotting speed of 8 – 10 mm s⁻¹, and dosing pressure of 80 – 100 kPa[27].

To achieve grid-like structures, successive layers were rotated by 90°. The scaffolds were plotted into 6-well plates in an air-filled plotting environment[27]. After plotting, the scaffolds were incubated in 0.1 M CaCl₂ solution containing 3 wt% sucrose for 10 min to crosslink the alginate. The scaffolds were then washed twice for 1 min with MS medium (Murashige and Skoog medium with vitamins) to prepare plant cell cultures. The dry weight of the inoculum was determined through the gravimetric analysis of reference samples (n = 9) after 48 h of drying at 60°C. Using 3D plotting and a novel
hydrogel blend based on alginate, agarose, and mc, basil cells could be embedded into the structured hydrogel matrix\cite{27}.

Most cells survived the fabrication process, according to microscopic analyses, live/dead staining, and metabolic measurements, and the cells could be cultivated in the plotted constructs. The novel alg/aga/mc blend demonstrated adequate printing and shape fidelity, potentially allowing the fabrication of constructs with open macropores in both vertical and horizontal directions under cell compatible conditions.

Therefore, the plant bioprinting technology presented in this study can generate a defined agglomeration matrix for plant in vitro cultures, allowing the acquisition of local and time resolved data as well as the control of the mass transfer and diffusion paths of substrates through the variation of macropore and strand dimensions. Bioprinted 3D cell cultures aid in the study of cell responses to environmental influences, including the replication of natural plant tissue for basic research. Bioprinting can thus improve existing methods through the application of immobilized cells in industrial biotechnology.

### 6.1.2. Novel food manufacturing using 3D-printed plant tissue

Plant cells and tissues are of particular interest for bioprinting because of their unique textural features, which are linked to both their porous microstructures and cell turgor pressures created by the cellulose-based CWs\cite{136-138}.

Pectin is an often used biomaterial for constructing matrix components because pectin serves as a binder between cells and comprises the middle lamella within plant tissues\cite{139,140}. Cells can be properly encapsulated and then printed at room temperature using pectin-based bioinks\cite{141}. Pectin is either low methoxylated (LM) or high methoxylated (HM) depending on the degree of methoxylation (DM)\cite{142}, which affects the gelation mechanisms. At low pH and high sugar concentrations, hydrophobic forces and hydrogen bonding generate HM pectin gels (DM >50%)\cite{143}. In contrast, LM pectin

### Table 4. Utilization of bioinks and algae cells in 3D bioprinting.

| Microalgal species | Hydrogel (Bioink) materials | Mode of application | Ref |
|-------------------|-----------------------------|---------------------|-----|
| Platymonas        | Silk fibroin - -            | Environmental applications on a small scale | [114] |
| Chlorella vulgaris| - - Kappa-carrageenan, chitosan, aloe vera, and Auro clay | Photosynthetic textile biocomposites | [131] |
| Chlamydomonas reinhardtii (strain cc125) | Alginate and Methylcellulose hydrogel-based bioinks | Effect of extrusion pressure and needle diameter on algal cell quantity after printing | [116] |
| C. reinhardtii    | Calcium alginate - -        | Photosynthetic living textile materials | [118] |
| C. reinhardtii (strain cc125) | Sodium alginate - - | Algae-cell hydrogel filters for removal of copper from contaminated water | [125] |
| Chlorella spp.    | Jeffamine polyglycol amines, poly (ethylene glycol) diglycidyl-ether and calcium-crosslinked alginate | (i) Embedding of various cells into the gelling matrix using 3D printing to form porous structures, (ii) Establishing optimal conditions for life and growth, as with most biological systems, can be difficult. Numerous plant or fungal products could be manufactured using a 3D-printed bioreactor technique rather than traditional plant growth, (iii) Photosynthetic gels | [135] |
gels (DM 50%) are produced by the production of calcium crosslinks between free carboxyl groups\cite{141}. The gelation mechanism of LM pectin is comparable to that of alginate with calcium, known as the “egg-box” model of gelation\cite{141}. Lamb’s lettuce cells (Valerianella locusta) were selected as the plant cells for encapsulation into pectin-based bioinks. Before isolating cells, a precise technique was followed, that is, Lamb’s lettuce (\textit{V. locusta}, (L) Laterr., var. “Gala”) was collected early in the morning from a commercial greenhouse under a 12 h light/dark regime at 150 \(\mu\text{m Em}^{-2}\text{s}^{-1}\). To remove dirt debris, the Lamb’s lettuce was cleaned with chlorine distilled water prior to shipment\cite{146}. The purchased Lamb’s lettuce was washed with 0.0005% NaOCl, rinsed 5 times with deionized water, and then dried before use.

The pectin solution was made using methoxylated pectin extracted from the peel of citrus and calcium chloride dihydrate\cite{141}.

D-glucose anhydrous, 4-morpholine ethane sulfonic acid hydrate, magnesium sulfate heptahydrate, bovine serum albumin (BSA), and pectinase from \textit{Aspergillus niger} were used as buffer solutions. Evans blue, fluorescein diacetate, Hoechst 33258, and pentahydrate (bisbenzimide) were employed to determine viability\cite{141}.

All pectin solutions used in this process were prepared the day before the lettuce cell extraction and maintained at 4°C.

To prepare the bioink, the pectin solution with encapsulated cells and a CaCl\textsubscript{2} suspension (in a 1:1 ratio) were mixed together\cite{141}. To modify the porosity and viscosity of the bioink, varied pectin and BSA concentrations were used to make 8 distinct bioinks with and without embedded lettuce cells. In total, 20 mL of pectin was prepared for this experiment. Before the final preparation of solution, 10 mL of the pectin solution was stirred for 10 min at 10,000 rpm, and air bubbles were stabilized using BSA. The solution was then agitated with a magnetic stirrer set at high speed. Lettuce cell suspension (2 \times 10 mL) was obtained by decanting 2 falcon tubes of raw suspension and washing with CaCl\textsubscript{2} solution (26 or 40 mM) overnight. The viability of the cell and CaCl\textsubscript{2} suspensions, which contained 13 or 20 mM CaCl\textsubscript{2}, was determined using the Evans Blue Exclusion staining procedure after decanting for 20 min. Then, the cell and CaCl\textsubscript{2} suspensions (5 mL of each falcon tube) were added dropwise to the pectin solution. When the solution became homogeneous, a gel formed almost immediately. Finally, the prepared bioink was lightly stirred for 5 min with a spatula, after which the bioink could be used for bioprinting. The viscosity and porousness of the ink can be adjusted by altering the pectin and BSA concentrations of the ink precursor, and the cell survival was unaffected by the formulation of pectin-based bioink processing and printing\cite{141}. Although pectin-based biomaterials are extensively utilized in food-based formulations, some chemicals used in this composition were not consumable. As a result, “bioink” is a more suitable name than “food-ink”\cite{141}. The color of the printed cubes changed depending on the bioink used; if the ink contains only pectin gel and no cells, the printed cubes will have a yellowish hue, while bioinks with lettuce cells usually produce a green hue, due to the presence of chloroplasts in the plant cells\cite{141}. Lightly-colored printed cubes usually were a product of the presence of BSA, which regulates the existence of air bubbles that induced transmission of light\cite{147}.

This method helps encapsulate land plant cells in high-density pectin gels with greater efficacy and consistency than previous methods\cite{141}. To date, 8 pectin-based bioinks, with and without cells, have been produced using this method. The framework introduced in this research can be viewed as the first step toward producing 3D-printed particulate or cellular foods.

6.2. Synthetic bioink

6.2.1. 3D bioprinting of artificial leaves

For the reproduction of the primary structural features of a leaf for quick mass transmission, 3D hierarchical macro/mesoporous structures with high surface areas must be synthesized\cite{148}. Titanium dioxide-based inks are ideal for creating 3D-printed leaf structures with high structural integrity and surface area as well as porosity-tunable 3D hierarchical constructions\cite{149} (\textbf{Figure 16}).

The ink was prepared by producing a sol-gel precursor solution called titanium diisopropoxide bis(acetylacetonate) (TIA), which contains two isopropoxide radicals surrounding one titanium atom, two acetylacetonate groups, as well as dodecyl benzenesulfonic acid (DBSA), which helps to improve the surface area. The pore volume was set to 0.1 cm\textsuperscript{3} g\textsuperscript{-1}, and the DBSA content is determined to obtain an optimal surface area consistent with that of typical mesoporous structures. However, producing TIA-based bioinks is often difficult because of diluted TIA suspensions, which are classified as low-viscosity Newtonian fluids, thus lacking the requisite rheological behavior for 3D printing\cite{150}. The DBSA-TIA ink should always sustain its cylindrical shape after ejection through a micronozzle, and it should have the capacity to span across spaces over underlying surfaces for large-scale fabrication of 3D structures\cite{151-153}. DBSA-TIA-based designs contain numerous woodpile layers, each of which is orthogonal to the one before it and connected by printed filaments spanning spaces between underlying surface structures.

There were other methods of bioprinting structures of micro leaves\cite{150}. Notably, the artificial micro leaf structure is similar to that of the macroporous architecture of a natural leaf, implying the possibility
of effective mass transfer\textsuperscript{[154]}. To achieve increased porosity and larger mesopores, the precursor was combined with silicon dioxide (SiO\textsubscript{2}) nanospheres after the SiO\textsubscript{2} is removed\textsuperscript{[154]}. The rheological parameters of SiO\textsubscript{2}-DBSA-TIA inks are identical to those of DBSA-TIA inks, indicating that it could be used for bioprinting. On combination with the SiO\textsubscript{2} nanospheres, the ink demonstrated shear-thinning and solidifying properties. Due to a higher solidness, the solidification slopes of SiO\textsubscript{2}-DBSA-TIA ink are much slower than those of DBSA-TIA ink\textsuperscript{[148]}. In addition, the SiO\textsubscript{2}/TIA mass ratio and SiO\textsubscript{2} particle size can be adjusted to create custom surface areas and pore size distributions; however, adding excessive amounts of SiO\textsubscript{2} can negatively affect ink homogeneity, making it impossible for the ink to flow.

Based on the optimized ink formula and taking into account both the surface area as well as rheological qualities, a well-patterned hierarchical macro- or mesoporous artificial photosynthetic system can be 3D-printed, wherein the leaf structure is obtained through calcination and etching\textsuperscript{[148]}.

Bioprinted 3D leaf structures can provide insights into the structure, function, and behavior, such as gas diffusion of plant leaves, thus contributing to advancements in the field of plant biology.

### 6.2.2. Bioprinting of plant cells for production of a biodefense agent

Transgenic rice cells were immobilized using this bioink (\textit{Oryza sativa})\textsuperscript{[155]}. This is the first report of recombinant plant cells being immobilized for the continuous synthesis of high-value heterologous proteins. The preparation of the suspension culture of rice cells is the initial step in this process. Encapsulation was performed using a transgenic \textit{O. sativa} rice cell line expressing recombinant rice butyrylcholinesterase (rrBChE)\textsuperscript{[4]}. Rice cells were cultured and grown in a semi-solid “NB+S” selection medium including N6 macronutrients, B5 micronutrients, and vitamins, 30 g l\textsuperscript{-1} sucrose, 1.8 g l\textsuperscript{-1} GelzanTM, 300 mg l\textsuperscript{-1} caseinhydrolysate, 250 mg l\textsuperscript{-1} L-glutamine, 250 mg l\textsuperscript{-1} L-proline, 2 mg l\textsuperscript{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.02 mg l\textsuperscript{-1} Kinetin, with 50 mg l\textsuperscript{-1} geneticin as the selection antibiotic\textsuperscript{[156]}. The cells were subcultured into liquid sterile NB+S media (without GelzanTM and geneticin) before the start of the experiments by pressing and sieving the calli through a sterile, stainless-steel, 280 m mesh sieve to achieve consistent cell aggregates\textsuperscript{[156,157]}. Suspension cultures were grown in 500 mL or 1 L shake flasks with a 20% working volume and incubated in the dark at 28°C, 140 rpm in a 19 mm circular orbit\textsuperscript{[157]}. The medium was exchanged weekly with fresh liquid NB+S medium. Cultures were kept in this state for up to 3 months before being replaced with new semi-solid-to-liquid subculturing.

The bioink used to immobilize the rice cells contained 12% (w/v) 4-arm polyethylene glycol tetraacrylate MW 20,000 (PEGTA), with 0.1% (w/v) LAP as the photo initiator. A 10× LAP stock solution and a concentrated PEGTA solution were made separately in NB+S medium and vortexed until completely dissolved to prepare the bioink\textsuperscript{[158]}. The LAP stock solution was added to the PEGTA solution immediately before the experiments began, and the mixture was vortexed again. The bioink formulation was prepared for demonstrating extrusion by adding nanocellulose crystal powder at a load of 16 wt% in a solution of 12% (w/v) PEGTA, 50 wt% cells, and 0.1% (w/v) LAP. Experiments were carried out in a biosafety cabinet with the bioink extruded manually using a 1 mL sterile Luer-lock syringe furnished with an ~840 μm inner diameter tapered tip. To reduce aggregation and assure a healthy exponential phase, growing rice cells in the suspension culture were received through a 280 μm mesh filter and passaged to fresh growth medium in a shaking flask few days before the start of the experiment\textsuperscript{[153]}. Before removing the media on day 0, 10 – 15 mL cell culture samples were obtained from the flask and gravity settled in a 15 mL Falcon tube. To obtain a 50% (w/v) cell loading density, cells were measured as grams of fresh weight (g FW) and added to the prepared bioink solution, or to fresh NB+S growth medium in the case of the liquid suspension culture controls\textsuperscript{[155]}. Using the Loctite EQ CL30 LED 405 nm Flood System with UV LED Flood Controller, cell-laden PEGTA-LAP bioink samples were cured under UV irradiation in a 6-well plate (3.48 cm diameter, 0.21 cm height, and 2 mL total volume) by exposing them to high intensity UV light for 10 s. At a 50 mm working distance, the lamp emitted a peak irradiance of 1500 mW cm\textsuperscript{-2} at 405 nm. A working distance of 21 cm was ensured between the lamp and the sample. To fit into the lip of a 25 mL flask, the cured rice cell-laden PEGTA-LAP hydrogel disks were cut into 3 – 4 pieces. The control conditions for liquid suspension cultures were performed with and without UV curing (Figure 17).

One gram of FW and 1 mL bioink (or 1 mL media for liquid suspension culture controls) was used to make cured cell laden PEGTA hydrogels, which were then introduced to 9 mL NB+S medium in 25 mL shake flasks. Flasks were incubated in the dark for up to 14 days at 28°C and 140 rpm.

The results suggested that this bioink (polyethylene glycol-based hydrogel) successfully immobilized transgenic rice cells (\textit{Oryza sativa}) producing recombinant butyrylcholinesterase, which acts as a prophylactic or therapeutic against cocaine toxicity.

\textsuperscript{4}The rrBChE promoter is controlled by the metabolically regulated rice alpha amylase 3D (RAmy3D) promoter, which induces protein expression in the absence of a sugar and contains a signal peptide that tags proteins for secretion.
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6.3. Hybrid bioink

6.3.1. 3D-printed bionic corals

Corals have evolved as specialized photon augmentation systems, resulting in space-efficient microalgal growth and exceptional photosynthetic quantum efficiencies\(^{16,159}\). Light attenuation (a decrease in the intensity of a light beam as it travels through matter due to the combined action of light absorption and scattering) caused by algal self-shading impedes the scale-up of microalgal cultivation processes. This barrier may be overcome by coral-inspired light management systems, potentially allowing large-scale bioenergy, and bioproduct production\(^{160}\).

This concept was used to create 3D printed bionic corals capable of hosting microalgae at spatial cell densities of up to \(10^9\) cells per mL. The hybrid photosynthetic biomaterials were created using a 3D bioprinting platform that accurately replicated the morphological features of living coral tissue and the underlying skeleton, as well as their optical and mechanical properties. As an outcome, the programmable synthetic microenvironment could mimic both the functional and structural aspects of coral-algal symbiosis\(^{160}\). In a dynamic environment with a limited resource base, evolution has enhanced the photosynthetic performance of coral, resulting in a high photosynthetic quantum efficiency, space-efficient light management, and high algal cell densities that approach theoretical limits\(^{161,162}\). While corals have evolved different geometries to achieve these results, they all comprise animal tissues that host microalgae and are built using a calcium carbonate skeleton that serves as a mechanical support and a scattering medium to optimize light delivery to otherwise shaded algal-containing tissues\(^{160,163}\).

The authors attempted to design a bioprinting platform capable of 3D printing living photosynthetic materials resembling coral tissue and skeletal source geometries, motivated by the optimal light control of corals.

Such discoveries can allow the use of coral-inspired biomaterials in coral reef conservation, coral-algal symbiosis research, and algal biotechnology.

Photopolymerizable gelatin-methacrylate (GelMA) hydrogel was used to produce bioink, and \textit{Symbiodinium} sp. was selected as the microalgae. In addition, another algal species, \textit{Marinichlorella kaistiae} KAS603, was used for growth observation (family Chlorellaceae)\(^{159}\).

The artificial coral tissue frameworks were created using a novel bioink solution that combined symbiotic microalgae (\textit{Symbiodinium} sp.) with a photopolymerizable gelatin-methacrylate (GelMA) hydrogel and cellulose derived nanocrystals (CNC), the latter of which provided mechanical stability and enabled the tissue scattering properties to be modified. A PEGDA-based polymer doped with CNC was used then to 3D print the artificial skeleton\(^{164}\). Following the printing procedure, a series of tests showed that the bionic coral induced the photon path length improvement approach of natural corals for avoiding algal self-shading\(^{159}\).

Then the microalgal cells were introduced into bionic coral to examine the growth of a free-living microalgal strain with an appropriate fatty acid profile for bioenergy generation. \textit{M. kaistiae} KAS603 grows in bionic coral with no flow and low incident irradiance (\(\text{Ed} = 80 \mu\text{mol photons m}^{-2}\text{s}^{-1}\)), reaching algal cell densities of \(>8 \times 10^8\) cells mL\(^{-1}\) by day 12\(^{159}\). This innovative bioink demonstrates outstanding biocompatibility for both free-living and benthic algae strains (Figure 18).

Therefore, bionic corals may inspire new fundamental biological research, motivate the development of synthetic photosymbiosis model systems, and lead to innovative solutions for efficient photon

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**Figure 17.** Plant cells can be printed in 3D. (A) Transgenic rice cells could be immobilized in increasingly complex topologies, including bioprinted lattices. (B) Extrusion results of a formulation with sufficient viscosity to extrude and preserve shapes with a high cell density. (C) A flow-through reactor with bioprinted plant cells entrapped inside. Reprinted with permission from Varma, A., Gemeda, H. B., McNulty, M. J., McDonald, K. A., Nandi, S., and Knipe, J. M. \textit{Immobilization of transgenic plant cells towards bioprinting for production of a recombinant biodefense agent}, John Wiley and Sons, Inc\(^{155}\).
augmentation in algal biotechnology\cite{159,160}. Furthermore, this type of setup can aid the investigation of natural coral morphology and incite translation to other contexts.

### 6.3.2. 3D printing of leaf-like structures for CO2 reduction

Liu et al. were the first to use cyanobacterial metabolism to recover CO2 in a hierarchically porous and transparent microsystem\cite{165}. A 3D architecture of a natural leaf with multi-scaled levels was imitated so that (i) water/nutrients could be transmitted to the bacterial cells through the botanic fiber layer (c.f. xylem and phloem), (ii) the cyanobacterial layer in the system could perform photosynthesis and respiration to decrease CO2 levels (c.f. mesophyll), and (iii) solar evaporation could help build up capillary force through the translucent and porous membrane layer (c.f. epidermis), potentially allowing self-sustaining capabilities. The authors developed a self-sustaining, biological artificial leaf that significantly lowered CO2 levels in the atmosphere and exchanged it with O2\cite{165}. Moreover, this artificial leaf structure can aid in understanding the natural leaf structure, water, and gas transport processes within natural leaves.

The preparation started with the culture of bacterial inoculum. The cyanobacterial stain *Synechocystis* sp. PCC 6803 was utilized for this procedure. *Synechocystis* spp. PCC 6803 was grown from glycerol stock cultures at 80°C by inoculating 15 mL of 5 BG-11 medium under gentle shaking with 12 h light and dark intervals. The BG-11 media consisted of 40 mg of K2HPO4, 1.5 g of NaNO3, 36 mg of CaCl2, 75 mg of MgSO4, 6 mg of citric acid, 1 mg of EDTA, and 6 mg of ferric ammonium citrate per 1 L of distilled water. For 2 weeks, a fluorescent lamp-controlled chamber offered continuous aeration and illumination at a temperature of 30 ± 2°C. The optical density at 600 nm (OD600) was used to track growth.

Membrane fabrication was the following phase, wherein silicone elastomer polydimethylsiloxane (PDMS) was combined with the curing agent at a 10:1 ratio and poured on top of the PMMA mold. The PDMS duplicate was peeled off from the PMMA mold for the suggested artificial leaf’s bottom reservoir after being hardened in an oven. The spin speed was carefully managed to establish a 40 μm gas-permeable layer on top of the PDMS membrane\cite{166}. Using a laser cutting tool, the botanic fiber layer was meticulously designed. Due to evaporation from sunlight, nutrients and water were transmitted from an external media reservoir to photosynthetic bacterial cells, while capillary force pushed the botanic fiber tube along, imitating the xylem and phloem of a natural leaf\cite{165}.

The sol-gel transition of the bioink (i.e., hydrogel-encapsulated cyanobacteria) must be carefully controlled for materials to retain their shape when patterned on the botanic fiber layer in this bioprinting method. In deionized water with 0.5 M CaCl2, cyanobacterial cells were encased in 6% (w/v) alginate.

The diffusion time required for the sol-gel transition increased as the calcium concentration increased, resulting in greater shear stress on the cells. As the rate of the reaction was slow, a partial uncross linked alginate layer could be deposited on the crosslinked layer; this bioink could be used to print several multilayered patterns. The printing was conducted using a 3D Vitarix™ bioprinter with the following settings: pressure: ~20 Mkpa; print speed: 25 mm s⁻¹, infill density: 30%; and nozzle size: 23 Gauge.

The final stage involved the bioprinting of the cyanobacterial cell-laden hydrogel on top of the botanic fiber layer and covering it with a gas-permeable PDMS membrane. The system generated a hybrid hierarchical cell/alginate architecture, with the gas permeable PDMS membrane encouraging gas exchange to the bacteria and botanic fiber layer supplying nutrients and water.

Figure 18. On Watakobi Reef, East Sulawesi, Indonesia, a colony of the coral *Stylophora pistilla* grows at a depth of approximately 10 m (A). Close-up shot of coral skeleton (B and C) and optical coherence tomography scanning of coral tissue (D and E). SEM view of a successfully 3D printed skeleton imitation, displaying corallites in 1:1 size to the original model (F). Growing *Symbiodinium* spp. microalgae on a living bionic coral (G). The bionic coral was cultivated for 7 days after the living tissue was printed on top of the skeleton imitation (from ref.\cite{159} licensed under Creative Commons Attribution license).
The bioprinted hydrogel-encapsulated bacterial layers were three-dimensionally bio-printed on the new botanic fiber layer and sealed with gas-permeable PDMS layers, emulating the structure of plants. Cyanobacteria convert $H_2O$ and $CO_2$ into carbohydrates and $O_2$ during photosynthesis, using the light energy and nutrients carried through the botanic fiber layers\textsuperscript{[165]}. During respiration, carbohydrates are digested to produce ATP for internal biological processes, while regenerating $H_2O$ and approximately half of the $CO_2$. This formulation is the only major strategy currently being seriously considered for novel $CO_2$ capturing technologies that rely entirely on heterogeneous photocatalysts. The proposed biological concept is capable of providing a more innovative, self-sustaining, scalable, cleaner, and significantly less expensive method of removing $CO_2$ from the atmosphere than current technologies\textsuperscript{[165]}.

A summary of the above-discussed bioinks is provided in Table 5.

### Table 5. Utilization of bioinks in the field of plant bioprinting.

| Bioink material | Mode of application | Ref |
|-----------------|----------------------|-----|
| Natural         | Synthetic            | Hybrid (Natural + Synthetic) |
| Alginate, agarose, and methylcellulose (hydrogel) blend | - | - | Production of a specified agglomeration matrix for the plant \textit{in vitro} cultures, which allows for the acquisition of spatially and temporally resolved data as well as control of substrate mass transfer and diffusion routes via macropore and strand dimension change | [27] |
| Pectin          | -                    | - | Creation of novel foods through 3D printing of plant tissue | [141] |
| -               | Titanium dioxide     | - | Understanding the artificial photosynthetic system and the underlying structure, function, and behavior of plant leaves through artificial leaf printing | [148] |
| -               | Polyethylene glycol-based hydrogel | - | Immobilization of transgenic plant cells for bioprinting to produce a recombinant biodefense agent | [155] |
| -               | Photopolymerizable gelatin-methacyryloyl, polyethylene glycol diacrylate | - | Bionic 3D-printed corals | [159] |
| -               | Silicone elastomer polydimethylsiloxane and alginate | - | 3D printing of a leaf structure to provide a more innovative, self-sustaining, scalable, cleaner, and significantly less expensive method of removing $CO_2$ from the atmosphere | [165] |

7. Future aspects and conclusion

Natural biomaterials will continue to be used for tissue engineering, with the goal of discovering and creating more complex morphologies, vasculature, and functioning tissue architectures that are commonly found in plant tissues. This goal can be accomplished by improving the mechanical, rheological, and biological properties of the biomaterial mixture employed in the production of bioinks\textsuperscript{[15]}; ECM-based bioinks laden with living cells show the promises in the production of 3D printed functional organs or tissues; however, this method can only be used in the production of a limited number of tissues and organs, as it requires large number of particular cells for these bioinks. Many other innovative biomaterials with stimuli-responsive hydrogels, supramolecular functionality, and reversible crosslinking polymers have recently emerged, which is promising in terms of bioink selection and usage.

Although fabrication has significant potential in plant science research, the printing process of plant tissue...
is not widely practiced due to a shortage of custom plant bioinks. Significant contributions of worldwide in the development of optimal bioinks present the opportunity for commercialized bioprinting technology and bioink in the future, which can be used in many fields, especially plant biology, for educational and industrial applications. In the future, the use of other materials and print heads to make more complex structures should be studied—for example, drug-filled microspheres can be added to bioinks to improve stem cell differentiation, survival, and cell viability\(^{167}\). Advancement in the understanding of plant morphology, function, and behavior is urgently needed to ensure future global food security, which necessitates technological advancement such as bioprinted plants. Although 3D printing in plant science remains in preliminary stages, the previous studies have demonstrated its potential for advancing plant science.

As 3D printing technology becomes more affordable and available, it will improve research coordination and scientific methodology\(^{31}\), and advance plant science and growth systems. Designs that are useful to plant scientists could be easily obtained using 3D printing without the need to be made commercially available. As this technology evolves in plant science, an open-source approach may be necessary for collective model improvement and adapting useful models to different plant systems. Thus, it is evident that in the field of bioprinting, encapsulating plant cells in biomaterials to create a bioink formulation could be effective in treating a wide range of human and other animal illnesses. In addition, bioprinted plant cells can facilitate the understanding of plant cell paracrine effects in \textit{in vitro} models of human and animal diseases.

In conclusion, the development and improvement of various bioinks is one of the primary paths for the advancement of green bioprinting. A greater diversity of bioinks will lead to a larger scope for green bioprinting.

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**Conflict of interest**

No conflict of interest was reported.

**Author contributions**

\textit{Conceptualization, investigation, writing-original draft, visualization, and review and editing:} Susmita Ghosh \textit{Supervise and guide:} Hee-Gyeong Yi

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