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Review article

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Trends in non-animal scaffolds for cultured meat structuration

Gabrielle Antunes Seibert[1], Vivian Feddern[2], Ana Paula Almeida Bastos[3], Anuj Kumar[4] & Silvani Verruck[1]

Cultured meat offers a sustainable protein alternative to meet growing food demands, relying on scaffolds to structure muscle tissue. This review evaluates plant-derived and polymeric scaffolds—synthetic polymers, peptides, fungal/plant materials—and techniques like 3D bioprinting, electrospinning, and microcarriers. Scaffold types (fibrous, porous, hydrogels) are assessed for adhesion, degradation, cost, and scalability. Highlighting cell compatibility, material pros/cons, and scalability challenges, the study identifies research gaps to advance cultured meat production.

The global population is approaching eight billion individuals and continues to rise daily. Projections indicate that by 2050, this figure will surge to between 9 and 11 billion people, precipitating a corresponding exponential surge in food demand[1]. It is known that meat consumption, conventionally obtained from animal production, is a fundamental part of the human diet. However, traditional meat production imposes several environmental and sustainability issues, including depletion of land and freshwater resources, greenhouse gas (GHG) emissions, and inefficient nutrient conversion[2]. The intensification of agricultural production is linked mainly to methane and nitrous oxide GHG emissions[2]. While agriculture contributes about 10–12% of total emissions, livestock production, especially ruminants, contributes around 14.5%[3]. Due to GHG emissions, public concern about climate change has arisen, with the need to feed the growing population. Consumer behavior is changing towards alternative meat sources, at least to some extent, as increased media coverage of climate change is associated with decreased demand for beef[2]. Despite increased awareness of climate change, global meat consumption continues to grow steadily[3], suggesting that the desire to avoid climate impacts is insufficient to reduce meat consumption significantly. Sustainability issues related to meat production, food-borne infections, diet-associated diseases, infectious diseases, and antimicrobial resistance are also some problems linked to the livestock sector[2]. Intensive livestock farming problems such as bovine spongiform encephalopathy and contamination of poultry, cattle, pigs, milk, and salmon products with resistant microorganisms, dioxins, and, more recently, swine flu and avian influenza[3] must be considered in the context of public health concerns.

For meat production to keep up with future demand, disruptive transformation, not only incremental improvements, is needed in food systems and their associated supply chains[4]. Thus, cultured meat (CM) emerges as an alternative source of protein that can meet future demand while addressing the challenges associated with traditional livestock[4]. CM represents in vitro biotechnological meat production without the necessity of animal sacrifice. More specifically, it is produced from cells using tissue engineering techniques. The strategy requires isolating some living animal cells by biopsy, followed by their expansion in bioreactors to produce a substantial cell mass content. This production mainly involves the generation of skeletal muscle tissue. However, CM also involves other cell types, including adipocytes for fat production, fibroblasts or chondrocytes to generate connective tissue, and endothelial cells for vascularization[5].

A few companies have already produced on the market, and many challenges need to be solved, such as high manufacturing costs and consumer acceptance related to the system's scalability[6]. In other words, CM production needs to be scalable efficiently, cost-effectively, and in sufficient volumes to meet market demand. The concern is that, even if it is technically feasible and meets quality standards, CM must also be produced at a scale that makes it affordable and price-competitive for consumers. Consumer acceptance will therefore likely depend not only on the product quality but also on the industry's ability to scale-up production to a level where CM can compete directly with traditional meat in terms of availability and cost. Additionally, it is important to achieve product characteristics that guarantee sensory and nutritional attributes equal, or better, to those of conventional meat products[5]. Significant technological challenges must be solved for this field to reach its full potential, such as establishing standardized cell lines, optimizing culture media, bioprocessing design, and scaffold technology[5]. Cell support scaffolds aim to resemble the structural and, to some extent, biochemical properties of the extracellular matrix (ECM) that support the structure and function of cells in natural tissues[6]. Consequently, various scaffold technologies, including electrospinning, edible cell microcarriers, texturized proteins, and 3D bioprinting, have been proposed to create 3D structures with suitable biological, structural, and mechanical characteristics. For its application in CM, however, the nutritional values of scaffolds and their safety should also be considered[6].

[1]Department of Food Science and Technology, Agricultural Sciences Center, Federal University of Santa Catarina, 88034-001 Florianópolis, SC, Brazil. [2]Embrapa Clima Temperado, BR-392, km 78, Monte Bonito, 96010971 Pelotas, RS, Brazil. [3]Embrapa Suínos e Aves, BR 153, km 110, 89715-899 Concórdia, SC, Brazil. [4]School of Materials Science and Technology, Indian Institute of Technology (BHU), Varanasi, India. e-mail: silvani.verruck@ufsc.br

Several factors need consideration when choosing biomaterials for CM scaffolds, including cell attachment, growth, and potential support for cell differentiation[7]. Biomaterials sourced from plants, algae, and fungi have emerged as attractive options due to their cost-effectiveness, enhanced safety profile, and high consumer acceptance. Moreover, leveraging knowledge from their processing in the food industry facilitates the adaptation of robust technologies for crafting these biomaterials into CM scaffolds[5]. Plant-derived proteins from legumes such as soy[8], chickpea[9], and carrot[7], as well as cereals such as zein (corn protein)[10], wheat[11], and oats[10], have been successfully processed into scaffolds using technologies like rotary jet spinning (RJS), stereolithography, electrospraying, electrospinning, and lyophilization, respectively. However, caution is warranted as these biomaterials might induce allergic reactions in susceptible individuals, necessitating precautionary allergen labeling[10]. Polysaccharides derived from plants and algae, including alginate[12], cellulose[13], and gellan gum[14], also present options for CM scaffold production. Despite their unfavorable nutritional profiles, these polysaccharides are deemed safe and can be tailored using various technologies to achieve desired structural configurations. Non-animal-derived biomaterials typically lack cell-binding domains essential for cell adherence and growth in culture, necessitating further chemical or structural modifications. Integrating biomaterials with RGD motifs or other integrin-recognized sequences can enhance cell adherence and initial growth[6]. Another possibility to ensure cell adherence to plant-based biomaterials can be through techniques such as 3D bioprinting, electrospinning, and extrusion, mimicking the 3D microenvironment of ECM in terms of structure and mechanical properties[8]. However, while these techniques are instrumental in creating biomaterials, they alone are insufficient to guarantee robust cell adhesion. To enhance cell attachment and promote successful integration with biomaterials, additional strategies, such as surface modifications, are often necessary[15]. These modifications have been extensively explored to reinforce interactions between biomaterials and cells. The properties of biomaterials are largely determined by the proteins adsorbed on their surfaces, which are critical in regulating cell adhesion, migration, proliferation, and differentiation[16]. Consequently, the regulation of protein adhesion on the surface of biomaterials is essential for optimizing their overall performance.

Polymers, synthetic or natural (biopolymers), are another choice for CM. They have shown applicability in numerous day-to-day life activities, including tissue engineering applications[17] and CM applications[13]. Among various polymer types, alginate (AG)[18] have shown potential in fabricating blends or composites for various applications, including scaffolds for CM and tissue engineering. AG is a hydrophilic biopolymer offering significant properties like non-toxicity, biocompatibility, biodegradability, and biostability that are explicitly suitable for cellular agriculture applications[18]. PCL has become popular in cartilage tissue engineering due to its low melting temperature, mechanical strength, biodegradability, and biocompatibility, suggesting a good approach for CM scaffolds[17,19]. Other polymers that have been used are polyurethane (PE)[16], polylactic acid (PLA)[20], polyvinylpyrrolidone (PVP)[21] and polylactic acid-co-glycolic acid (PLGA)[18]. Although these polymers have been used, they need additional removal steps at the end of the process, which can lead to higher manufacturing costs and add more complexity for the process[10]. Polymeric scaffolds possess unique properties such as high surface-to-volume ratio, high porosity with very small pore size, suitable biodegradation rate, and mechanical properties. The advantages of these scaffolds are the versatility of chemistry and biological properties, which are noteworthy for tissue regeneration. Synthetic polymers also have a major advantage over other materials because they can be produced in large, uniform quantities and have a long shelf-life[16]. On the other hand, the disadvantages are specialized polymers and biomaterials for regenerative medicine can be expensive, what would make CM economically unviable on a large scale. Scaffolds in regenerative medicine are designed to support cell growth and regeneration of complex tissues in the human body. Adapting them for CM production requires complex modifications, for instance, structural and functional composition (such as texture and taste), which could compromise the final product quality. Also, even if scaffolds are biocompatible and safe for medical use, they need to meet specific food safety regulations, which may require additional testing and delay market entry. Different countries may have different regulations on the use of scaffolds in food products, creating additional challenges for CM internationalization. Finally, regenerative medicine application technology to food may face resistance from consumers, who may have concerns about the safety or naturalness of the product. The materials used in medical scaffolds may not be sustainable or may have a significant environmental impact, which contradicts the proposal of cultured meat as a more sustainable alternative to conventional meat. The compatibility of scaffolds with the culture media used for cell growth in cultured meat may be limited. Medical scaffolds may not be optimized for the cell growth environment required for meat, which may lead to inefficiencies in the cultivation process.

For this reason, this review aims to describe the main non-animal scaffolds based on polymers or plant, bacterial, or fungi-derived materials that have been tested, addressing the methodologies used for structuring CM. In addition, the recent methods used to create scaffolding material will also be discussed.

Production process of cultured meat

The production of CM is inherently dependent on the generation of muscle tissue, as it serves as the foundational element for this innovative food source. Therefore, it is necessary to know the structure of natural muscle tissue to develop applications with scaffolds for CM through tissue engineering technology. Vertebrates have three classes of muscles: skeletal, smooth, and cardiac. For CM, skeletal tissue is mostly used since it is generally well-preserved among species[22].

Mature muscle fibers are very long compared to other cells in the body. They are multinucleated, with up to 100 nuclei[23] packed in myofibrils, bundles of contractile filaments composed of long chains of actin and myosin. The filaments are divided into functional contractile units called sarcomeres. Overlapping actin and myosin within myofibrils gives muscles their characteristic striated appearance[23]. These muscle fibers are in dense connective tissue composed of molecules of the ECM. The distinctions between endomysium, perimysium, and epimysium are based on microscopic observations. These differences in the molecular composition of the three layers suggest that the endomysium, which involves the muscle fibers, has a mechanically stronger collagen network than perimysium and epimysium[24]. This muscle stiffness is 2–12 kPa, which may benefit cell expansion, and increased tissue stiffness could induce differentiation[25]. However, the structure is heterogeneous, and stiffness variations may occur, which impacts the product's final texture and softness. The ECM profoundly affects meat quality, related to its biological effects on living tissue and even changes during post-mortem. The main components of the ECM are collagen, proteoglycans, and glycoproteins[25].

Collagen is a fundamental protein in the structure of muscle tissue. Its crosslinking plays a significant role in mechanical properties and may vary according to muscle type, species, and age[25]. It is important to note that muscles with higher collagen content may be less nutritious due to the increase in glycine, a non-essential amino acid[23], and the absence of the essential amino acid tryptophan[26]. In chickens and pigs, it is believed that differences in collagen levels do not significantly impact sensory quality within a typical range[23]. This occurs because most animals bred for consumption are slaughtered at a young age when collagen crosslinking levels are weak. On the other hand, fish muscle collagen has low thermal stability, resulting in structure loss during cooking. This phenomenon is responsible for the scaly texture of cooked fish due to collagen fusion. Thus, scaffolds must be produced with myofibrillar proteins in order to achieve the desired texture in the final cooked product[23].

Skeletal muscle development begins with the proliferation of myoblasts, muscle precursor cells that multiply and migrate with the help of ECM components such as fibronectin and collagen. Upon exiting the cell cycle, myoblasts begin to differentiate and fuse to form multinucleated myotubes, a process in which ECM proteins such as laminin and type IV collagen provide the necessary structure (Fig. 1). During this differentiation

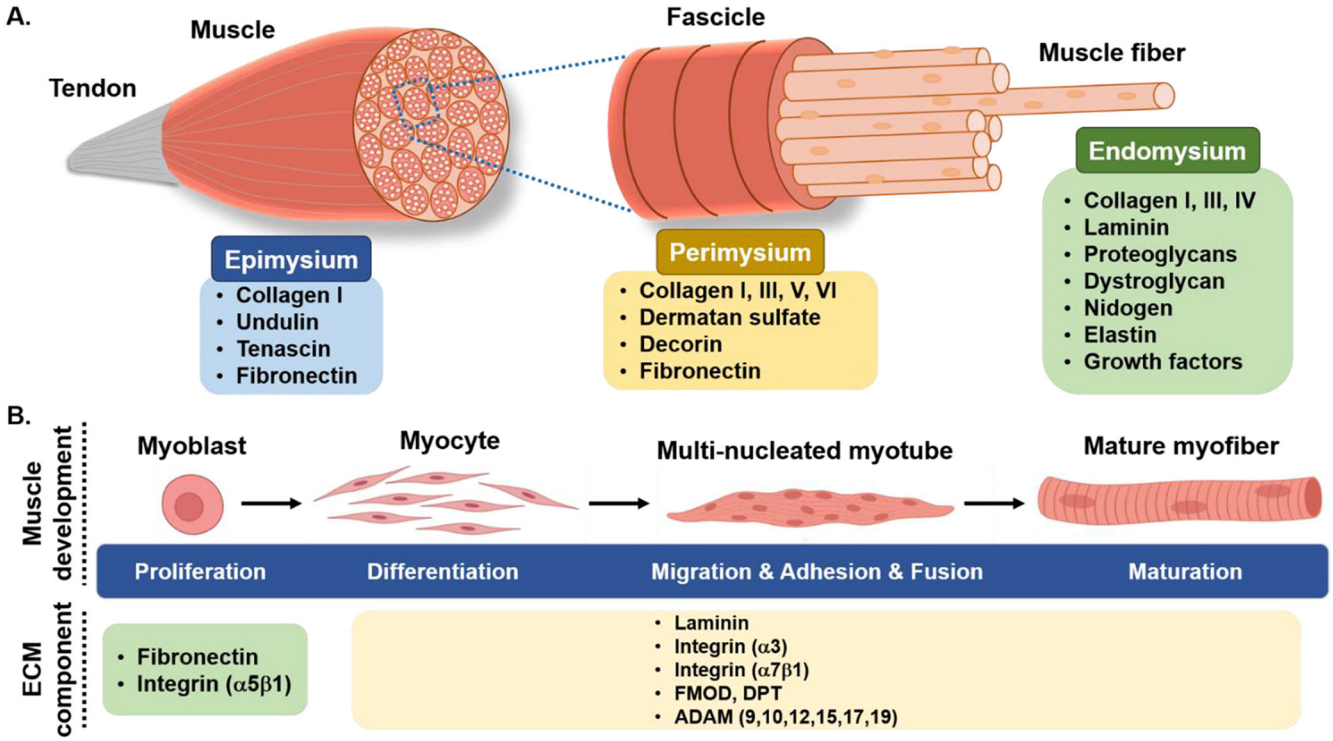

Fig. 1 | Skeletal muscle ECM. A three-layer structural schematic and associated ECM components. **B** Schematic representation of ECM-related protein activities during the different phases of myogenesis. Reprinted with permission from Ahmad et al.[123].

phase, metalloproteinases remodel the ECM to accommodate myotube growth, while proteins such as tenascin-C and decorin modulate cell adhesion and growth factor activity. In the maturation phase, myotubes develop into functional myofibers, with ECM components continuing to stabilize muscle fibers, organize the contractile machinery, and maintain muscle function[20]. The dynamic interaction between the ECM and cells is essential throughout all these stages, ensuring the proper formation, alignment, and functionality of mature myofibers.

In addition to collagen, proteoglycans, and glycoproteins play an important role in muscle ECM. These macromolecules comprise a carbohydrate-bound protein, and proteoglycans contain hydrated glycosaminoglycans, such as heparan sulfate and dermatan sulfate[27]. Proteoglycans contribute to the binding between the basement membrane and collagen in the endomysium and act in the sequestration of growth factors. The most common proteoglycans in muscle tissue are dermatan sulfates and chondroitin sulfates, such as decorin and biglycan[27]. Glycoproteins, such as fibronectin, laminin, and nidogen, have oligosaccharides and play a role in the connection between the cell membrane of muscle fibers and the basement membrane. They connect directly to integrins on the cell surface and collagen IV in the basement membrane, forming a complex and interconnected structure in the basement membrane[27]. Considering the complex ECM structure for muscle structuration, developing scaffolds that mimic all the ECM structures is one of the main challenges in the CM production process to be surpassed. Figure 1 shows the muscle structure.

The CM production process can be divided into three steps[27]. Figure 2 illustrates the three steps before the industry processing. The first step involves obtaining cells that can differentiate into various types, such as muscle cells (myocytes), fat cells (adipocytes), and fibroblasts, necessary for meat composition requirements[27]. The initial step is based on isolating and characterizing the appropriate cells of the species of interest and storing them. This step often covers the development of a stable and immortalized cell line[27].

The second step focuses on developing growth media that enable high cell proliferation rates from safe and low-cost food-grade ingredients. In this step, the cells are expanded to increase total biomass. The goal is to produce many cell duplications, keeping the cells undifferentiated and proliferative. For example, cells can be grown in a stirred bioreactor or spinning flasks in microcarriers as aggregates or individual cells[28]. Bioreactors and bioprocesses must be designed to meet the constraints of cost, sterility, food safety, and the ability to maintain appropriate conditions for cell growth and tissue maturation in the long term; these considerations are better discussed in the bioreactors section.

The third step concerns the development of food-safe scaffolds that mimic the function of the ECM[25]. In this step, which corresponds to tissue maturation, cells are grown under conditions that promote cell differentiation and maturation, usually but not always, in scaffolds[25]. The choice of medium and bioreactor is crucial in steps 2 and 3 and probably differs between both[28]. Further processing must be done to transform engineered tissues into different product types. For example, scaffolds loaded with mature myofibers can be combined with edible microcarriers, in which adipocytes are differentiated in step 3 and separated from the original process to form a hamburger.

Although growing mammalian cells in stirred-tank bioreactors with up to 20,000 L capacity in the pharmaceutical industry is already possible, significant advances in bioreactor projects are still necessary to support the large-scale production of structured tissues[29]. Depending on the final product and the chosen process, additional processing steps may be required to transform the cells or tissues into the final product, which may be similar to the methods used in conventional meat production[22]. Alternatively, several types of cells can be differentiated in different media before their insertion into the scaffolds to make up the final product. Self-assembled cell sheets can also be used, in which the secreted ECM proteins become the scaffold itself[30]. Further variations in this general scheme may combine steps 2 and 3 by performing tissue maturation on a larger scaffold processed directly like conventional meat[22].

Scaffolds of non-animal origin

Self-assembling peptides represent a promising class of non-animal-derived scaffolds for cultivated meat applications. Their intrinsic ability to mimic the ECM, mechanisms of self-organization, innovations in controlling cell

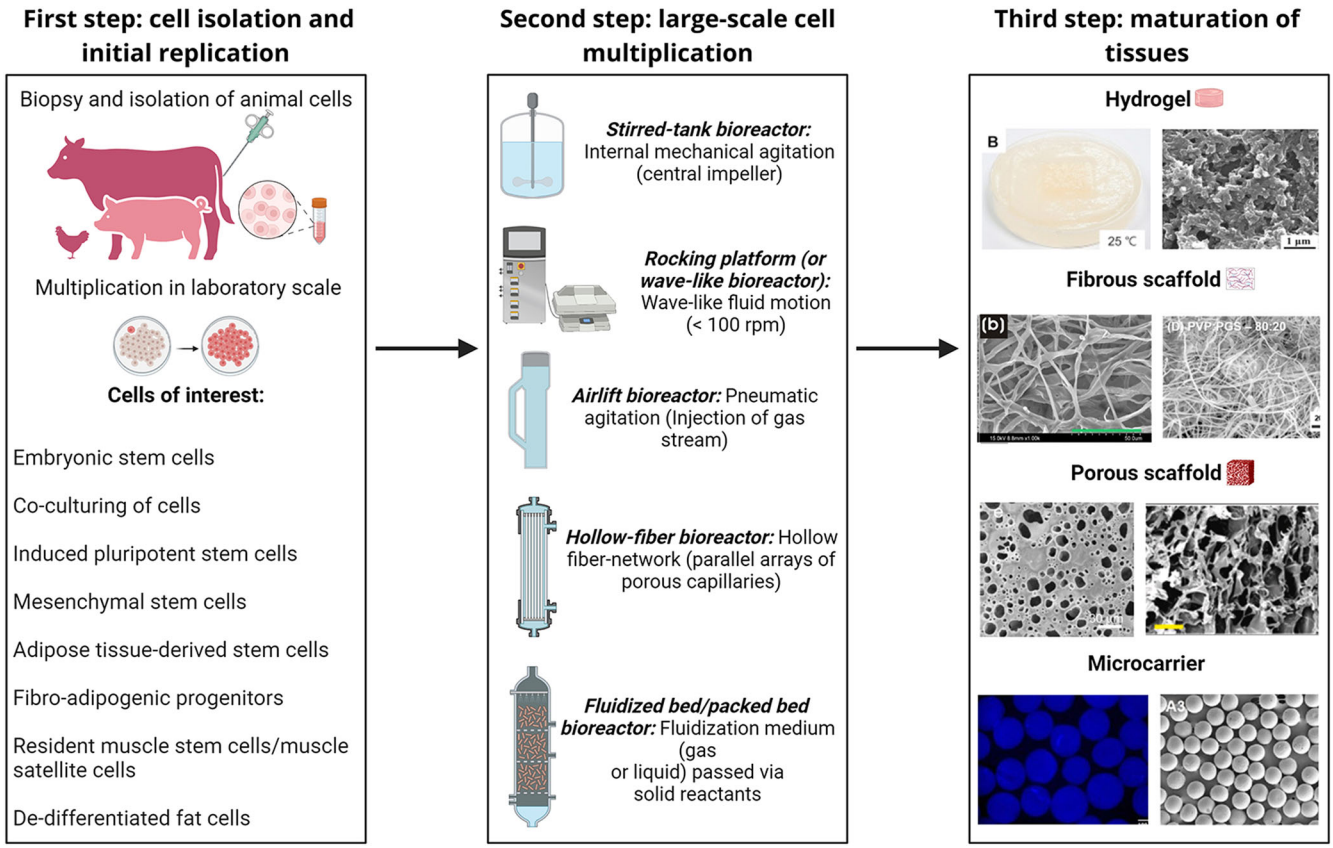

Fig. 2 | Schematic production process for CM and scaffold types. Reprinted with permission from refs. 7,9,11,17,21,29,30,52, Copyright 2024 Elsevier.

adhesion, and the ongoing efforts to overcome cost barriers. These factors make self-assembling peptides a viable and innovative option for scaffolding in cell agriculture, potentially transforming how cultivated meat is produced and scaled in the future. Choosing a scaffold suitable for CM depends on the material's properties, considering the cell-adhesion properties, cost, and degradation profile[5]. Synthetic polymers, self-assembling peptides, ECM molecules, and plant, bacteria, algae, and fungal-derived materials are being used[2], although they can be combined to obtain a product that contains the advantages of each material[130]. Scaffolds produced using different approaches focusing on polymer-derived biomaterials and biomaterials derived from plants and fungi are shown (Table 1). Therefore, these materials can be utilized individually or in combination to create a product that capitalizes on the strengths of each component. For example, combining various materials can improve the final product's strength and flexibility, replicate the ECM found in natural tissues, enhance protein content and nutritional profile, promote cell adhesion and differentiation into muscle, optimize cost efficiency, and refine organoleptic properties such as texture and appearance to closely resemble traditional meat.

Synthetic polymers offer a wide range of advantages for cellular agriculture. The possibility of precise adjustments in their physical and chemical properties allows them to be adapted to the specific needs of each application[31]. However, it is important to note that these polymers are not found in nature, which makes manufacturing in specialized laboratories essential[31]. Although they have advantages, many are not edible and have slow degradation rates[31]. This creates the need for a costly step of cell dissociation from scaffolds after the cell proliferation phase[5]. While increasing the production cost, this step is essential to ensure that cells can be released from the scaffold and used to form functional tissues[5]. Despite this challenge, in some cases, the advantages provided by certain synthetic polymers, such as PLA[20], PLGA[32], and PCL[30] can be considered for scaffold applications. Studies indicate that it may be possible to establish faster degradation scaffolds to use as tissue-embedded structures in certain configurations if degradation products are not harmful to cells and do not remain in the final CM[22].

The mimicry of ECM properties is one of the main objectives in developing effective scaffolds. Properties such as its mechanical strength and flexibility, its effects on cellular behavior, and its nutrient composition must be considered. The complexity of ECM can be overcome by scaffolds containing one or more of the main structural proteins, developing growth factors, transcription factors, and cytokines to stimulate normal cellular behavior and secretion of ECM[31]. Substituting proteins derived from animals for proteins produced by microbial fermentation, plant molecular cultivation[33], or cell-free systems[24] is a promising option. When using proteins produced by these methods, it is possible to ensure the biocompatibility of scaffolds and their efficient integration with cells, which is crucial for cell agriculture's success. Animal-free collagen, for example, has been successfully produced by plants, bacteria, and yeasts[29]. Bacterial collagen is particularly advantageous as it lacks the limitation of coexpression with enzymes, which facilitates its production and enables its use on a large scale[34].

Some species of bacteria and algae also emerge as promising alternatives for developing scaffolds. These natural raw materials can produce cellulose, a key component in creating scaffolds[35]. Bacterial cellulose-based structures have been widely evaluated for biomedical tissue engineering applications, revealing themselves as potential scaffolds for CM[35]. A study on bacterial cellulose synthesized from *Gluconacetobacter hansenii* (ATCC 23769)[31] evaluated the synthesis, characterization, and potential applications of bacterial cellulose (BC) hydrogels[36]. The biocompatibility of hydrogels was evaluated using endothelial cells of immortalized human veins (HUVEC), and the results showed that the hydrogels were biocompatible and showed sufficient mechanical resistance for handling and replacing organs or their parts. The research evaluated the potential application of BC hydrogels in tissue engineering, but no possible uses for cell agriculture were addressed[36]. On the other hand, fungal by-products, such as

Table 1 | Plant, fungi-based and polymer scaffolds with suitable application in cellular agriculture

Scaffold nature/Material	Mechanism	Cell line	Scaffold processing details	Ref.
Hydrogel Cellulose acetate nanofibers (CAN)	Electrospinning/Film stacking process	C2C12 and rat cardiomyoblasts (H9c2)	CA was dissolved in acetone-DMF (3:1 v/v) to obtain a 12% w/v solution. CAN was achieved by electrospinning under fixed parameters: voltage (16 kV), collecting distance (14 cm), collector rotation (400 rpm), and solution gravity-fed at room temperature. The collector rotation had two parameters: 400 rpm (film formed by nanofibers deposited with random orientation) and 1500 rpm (nanofibers' deposition aligned in parallel). The nanofibrous membranes were dried (vacuum chamber) for 3 days to remove residual solvents.	13
Microcarriers Hydrolyzed protein of chickpeas	Electrospraying, alginate microbeads coated with a variety of protein hydrolysates	C2C12, porcine myoblasts, chicken satellite cells, and 3T3-L1	After enzyme inactivation, electro-pulverized alginate microbeads were coated with various protein hydrolysates: mung bean, soy, red lentil, chickpea, pea, and rapeseed (two types). After coating, the microspheres were collected and triple-washed.	9
Microcarriers Broccoli florets	Decellularization	Primary bovine satellite cells	Broccoli florets (1 g) were placed in a 50 mL tube (45 mL deionized water + sodium dodecyl sulfate 10%w/v + 3% tween-20, 10% bleach), stirred/48 h and refreshed after 24 h. A decellularized solution was aspirated from the samples, and the florets were transferred to a 2 L beaker (with deionized water) for at least one hour. Samples were stored in deionized water at room temperature.	85
Porous scaffold Glutenin wheat powder	Crosslinking method based on water annealing	C2C12 and bovine satellite cells (BSCs)	Glutenin powder was dissolved in water and then distributed in 48-well plates (0.5 mL/well) to perform lyophilization. Glutenin solutions (2.5 and 5%w) prepared at pH 3 were transferred to molds in which a steel sheet separates one side ($3.5 \times 2.5 \times 1.5\ cm^3$) from the other chamber side ($3.5 \times 5.5 \times 1.5\ cm^3$). Solutions were added to one side of the steel sheet and N_2(v) to the other to generate freezing of the glutenin solution. They were frozen and then lyophilized at –80 °C. Dried fibrous glutenin scaffolds with directionally aligned pores were autoclaved (121 °C/15 min and dried/10 min).	11
Hydrogel Callus-based food inks (CBF)	Stereolithography	Plant cells	Scaffolds were 3D printed by stereolithography (length/width 40 mm; height 3 mm) using the reversible free-form incorporation of hydrogels in suspension. Bioinks of CBF in alginate for cell content (1:2, 1:1, and 2:1w/w) were loaded in a 30 mL syringe and then printed on 3D network scaffolds in a gelatin paste support bath. The gel structures were soaked/1 h to allow the alginate in the bioink to cross-link with the calcium ions in the bath. Scaffolds were collected and washed 3× with 1/2 MS. The parameters were layer height (0.9 mm), nozzle diameter (1.0 mm), nozzle speed (20 mm/s), and filling level (60%).	7
Hydrogel In vitro, the cell culture of basil combined with AG, agarose, and methylcellulose (AG/AGA/MC)	EBB	Plant cells	Hydrogel bio-inks were dispensed through a conical needle dosage, with an internal diameter of 610 µm, applying a plot speed of 8–10 mm/s and a dosage pressure of 80–100 kPa. Successive layers were displaced at 90° to obtain grid structures. The scaffold was plotted in 6 air wells as a plot environment. After the plot, the alginate crosslinking was obtained by incubating the scaffolds in 0.1 M $CaCl_2$ solution containing 3%w sucrose/10 min. Subsequently, the scaffolds were washed 2×/1 min in MS medium for plant cell cultures.	12

certain polysaccharide fractions found in leafy *Grifola*, have shown beneficial effects beyond their role as supporting structures[37]. Another study has shown that these fractions stimulate collagen proliferation and synthesis in fibroblastic cells, making them a promising option for creating innovative and effective scaffolds for cell agriculture[38].

Another promising approach in cell agriculture involves self-assembling peptides. These peptides are studied as scaffolds for structural support and materials used in 3D bioprinting. The highly versatile nature of self-assembling peptides allows them to self-organize into complex three-dimensional structures, simulating the ECM. This property has enormous potential to create scaffolds that mimic the structure and function of animal tissues. Two mechanisms are known for the self-assembly process[39]. The first is the differential adhesion hypothesis, based on cell-cell bonding behavior and free energy minimizations, which drive self-assembly[40]. This interaction occurs because of the adhesion of proteins on the cell surface, creating a cell mass that moves together with a liquid, reducing its surface tension[40]. Cells with high surface tension tend to move to the center to improve intercellular adhesion, and similarly, in the self-assembly process, the non-adherent substrate feeds the cell population consistent with intercellular adhesion, leading to the minimization of free energy[41]. The second mechanism is based on the differential interfacial tension. The difference between this method and the previous one is the minimization of free energy, which is the cellular behavior, and not the substrate, with cell movement governed by forces created by the cell cytoskeleton in the cell membrane[39]. Cells with similar interfacial stresses will aggregate, while cells with different interfacial tension tend to remain separate[42]. An important advance in this area is the development of peptide coatings designed to control cell adhesion and detachment[43]. These coatings combine peptide sequences, such as arginyl-glycyl-aspartic acid (RGD), which promote cell adhesion with cleavage sites, allowing the controlled release of cells and, consequently, the continuous production of cells[43]. Innovative companies are leading this research and driving the use of these self-assembling peptides in practical applications. However, it is important to mention that the costs associated with manufacturing these peptides still pose a significant challenge for their large-scale adoption. Also, peptide scaffolds may not be suitable for long-term CM bioreactor culture, due to their generally poor mechanical properties. In this sense, optimizing current techniques and using recombinant organisms may be crucial to make these peptides viable in scaffolds for ECM[44]. Advancements in needle-free and multi-needle electrospinning technologies have demonstrated that the process can be scaled to industrial levels, resulting in a substantial increase in production rates[45]. Depending on the polymer and process parameters, industrial-scale electrospinning systems, such as needle-free systems, can attain production rates of up to 1 kg/h or more. Additionally, recent advancements in high-throughput electrospinning machines have made it feasible for commercial manufacturing to produce continuous fibers on a large scale. The potential for large-scale production of electrospun materials is underscored by these advancements, which bolster their viability for industrial applications.

Scaffolding technologies

Several techniques can be employed to achieve scaffolds, such as 3D bioprinting, electrospinning, microcarriers, and decellularization. Scaffold-free approaches are explained in this review as well. These techniques are presented in Fig. 3.

Crosslinking can be achieved through physical, chemical, or enzymatic means to form the scaffolds. Physical crosslinking arises from physical interactions, including ionic interactions, temperature-triggered mechanisms, and dehydrothermal crosslinking (DHT)[39]. Ionic interactions involve crosslinking agents forming ionic bridges with the polymer backbone[46]. Temperature-triggered crosslinking relies on thermal behavior to form crosslinks, while DHT involves subjecting the polymer to high temperatures under vacuum to remove water and create crosslinks[47]. Xiang et al.[11], investigated physical crosslinking through steam sterilization or water annealing to create porous glutenin sponges and fibrous aligned scaffolds to support the proliferation and differentiation of C2C12 mouse skeletal myoblasts and bovine satellite cells (BSCs). These scaffolds obtained pore sizes ranging from 50 to 250 μm and showed good cell adhesion and proliferation without RGD motifs or the addition of extra ECM protein coatings. Also, the study demonstrated physical crosslinking methods based on hydrogen bonding, which resulted in structural stabilization due to the formation of β-sheet crystals[11]. Physical crosslinking could improve food

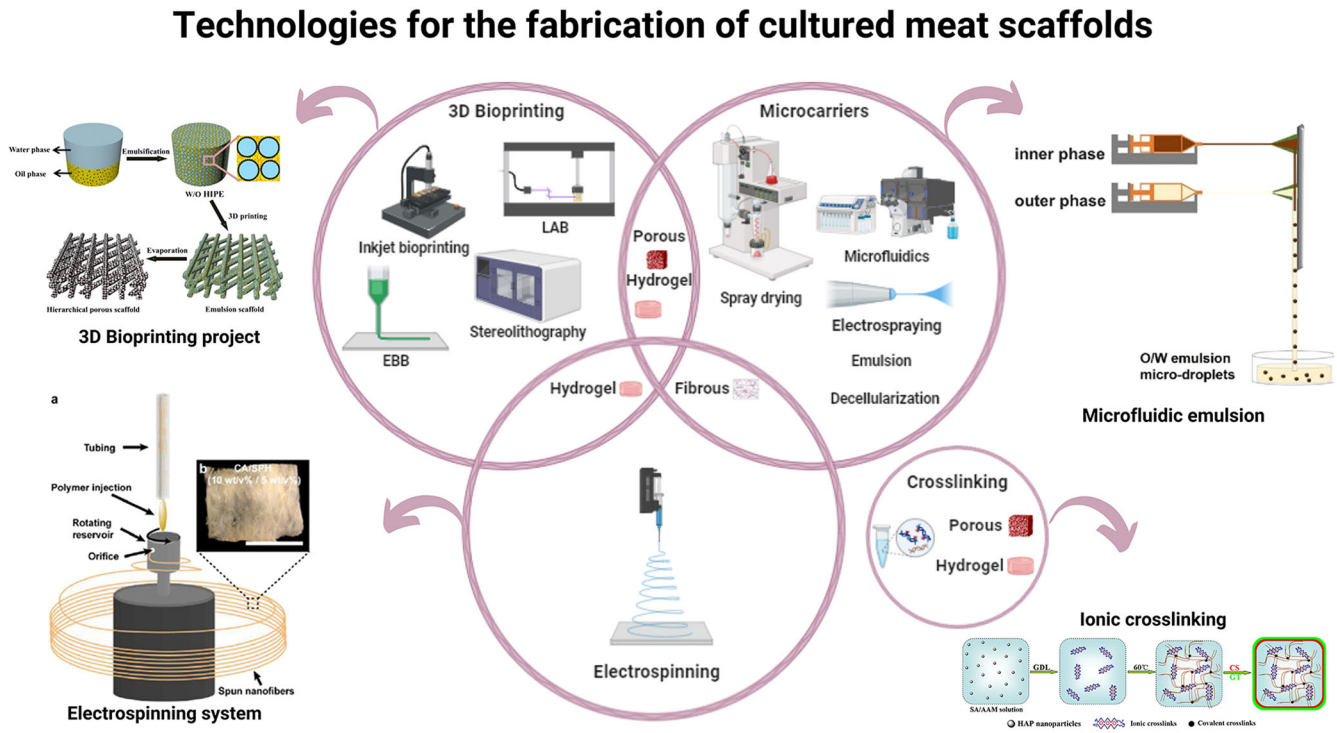

Fig. 3 | Methods for the fabrication of cultured meat scaffolds. Reprinted with permission from refs. 8,17,18,30. Copyright 2024 Elsevier.

safety by avoiding additives compared to chemical crosslinking for structural stabilization.

Chemical crosslinking typically involves covalent bond formation with polymer chains during scaffold synthesis. Chemical crosslinking creates more stable scaffolds than physical methods[39]. Over time, several small crosslinking molecules, like genipin, dopamine, glutaraldehyde, and tannic acid, have become popular for scaffold synthesis. Although chemical crosslinking can achieve more stable scaffolds, they have some toxicity problems that should be considered. For example, glutaraldehyde (GTA)[48] which is widely used in biological sample preparation, disinfection, and as a crosslinker for proteins and biomaterials, is associated with irritant and sensitizer, it can cause respiratory issues and skin irritation. Epoxy compounds (e.g., ethylene glycol diglycidyl ether), used to crosslink proteins, DNA, and other biomolecules may cause respiratory irritation, skin sensitization and carcinogenic effects. Diisocyanates (e.g., methylene diphenyl diisocyanate (MDI)), are used to produce polyurethanes and as crosslinkers in various industrial processes, causing occupational asthma, chronic lung disease, and skin irritation[48]. Acrylamide and N,N'-methylenebisacrylamide (MBA), used often together to crosslink gels, are considered hazardous (neurotoxic) because they are linked to cancer[6]. Another common example of chemical crosslinkers associated with toxicity is formaldehyde[39]. While chemical crosslinkers are essential for creating stable scaffolds, many of them pose significant toxicity risks, affecting cell viability and human health. Alternatives, such as using less toxic crosslinkers or non-chemical methods (e.g., enzymatic crosslinking), are being explored to reduce these risks in applications requiring high biocompatibility. Conventional chemical crosslinking methods, such as photo-crosslinking and covalent crosslinking, lead to scaffolds with enhanced stiffness and rapid gelation times. To address the issue of cytotoxicity, enzyme crosslinkers have gained popularity, providing a potentially safer alternative and enabling a better microenvironment for artificial scaffold development. An example of chemical crosslinking involves using glutaraldehyde, to create glucuronoxylan-based quince seed hydrogels. The porosity of these hydrogels was measured before and after crosslinking. In the non-crosslinked scaffolds, the average pore size was about 99.85 μm with 22.52% porosity. After light crosslinking, the average pore size decreased to 76.59 μm with 18.36% porosity. The average pore size decreased to 56.04 μm for heavily crosslinked samples, with 13.58% porosity[48]. This indicates that increased crosslinking creates a denser scaffold, reducing interconnected pore size and porosity[48]. An interconnected and porous structure is a critical aspect of scaffold design[48], showing that the porous glucuronoxylan-based quince seed hydrogel has the potential for cellular agriculture applications.

3D bioprinting

First used in 1986 by Charles W. Hull, 3D bioprinting is a process that consists of creating a layer-by-layer model from a computer development[2]. 3D bioprinting can be done using different methods: inkjet, laser-assisted bioprinting (LAB), laser-induced forward transfer (LIFT), EBB, and stereolithography[37] (Fig. 3).

The different 3D bioprinting approaches require appropriate bioinks optimized to ensure the cellular fidelity of the printed scaffold while supporting cell viability. 3D bioprinting technology offers several advantages for food production, including the ability to customize food products' shape and composition. This technology also enables the fortification of foods, improving their nutritional profile to better meet specific dietary needs[37]. Applications of 3D printing in food production include the creation of innovative shapes and complex geometries, such as structured cultured meat that resembles steaks[49]. One of the current challenges with texture-modified foods is that the processes used to achieve a safe and desirable texture often compromise nutrient density and result in a lack of visual appeal, which can negatively impact appetite. In contrast, additively manufactured foods, due to the precision of the extrusion process, can achieve the desired texture, improve the nutritional profile, and offer a more visually appealing presentation. However, there is currently no research available on the nutritional quality, potential nutrient loss, or nutritional stability of these food products, particularly when production is scaled up. Bioinks used in food include biopolymers such as gelatin[7], agarose[12], cellulose[8], alginate[7], pectin[50], and plant proteins such as soy[51]. These biopolymers have crosslinking mechanisms, allowing the formation of a stable hydrogel in the printed construction, while the bioink maintains its desired fluid properties. These polymers may also undergo thermal crosslinking, like in agarose or pH-based gelling, such as pectin. Cellulose can be cross-linked in different ways, such as UV radiation, enzymes, or calcium ionization[12]. Another consideration that should be made in CM 3D bioprinting is the stability of the printed structure during further processing and the cooking processes[11]. The versatility, precision, and reproducibility of 3D bioprinting show that it is a promising method for CM production (Table 1).

Although 3D printing has been demonstrated for various materials, the most relevant bioinks to CM are hydrogel-based[10,12,18,48,52]. A hydrogel is a hydrophilic polymer matrix crosslinked by physical or chemical means and has a water retention capacity. Hydrogels are very important in cellular agriculture and must have indispensable requirements to be applied as scaffolds. For example, the polymer matrix must be cytocompatible and contain non-toxic biomaterials[12]. Micronutrients and signaling molecules must also be able to reach cells throughout the tissue, and for this, the hydrogel diffusion kinetics must allow these molecules to penetrate the entire hydrogel thickness at the concentrations and rates required for support cells[12]. Stiffness is an important factor for a hydrogel, as it can affect cell motility, proliferation, differentiation, and migration[53] since cells must be able to reshape the hydrogel during tissue maturation. Finally, the degradation rate of the hydrogel should align with the cells' ability to remodel their microenvironment and deposit ECM components to compensate for scaffold loss. For example, the referenced study[54] achieved a maximum ECM area of 61.08 mm^2 using a 75/25 PLGA/collagen scaffold seeded with C2 cells. Additionally, proteolytic sites should be incorporated into the hydrogel to facilitate cell adhesion and migration.

Hydrogels, despite their potential for applications in CM due to their ability to mimic the ECM and support cell growth, have some potential disadvantages. A major concern is in their mechanical properties; hydrogels typically lack the necessary strength and rigidity to fully replicate the texture and structural integrity of traditional meat, which can impact the whole consumer experience[55]. Furthermore, the biocompatibility of certain hydrogels might provide challenges, as not all hydrogel materials are suitable for food-grade applications, necessitating careful selection and potential modification to ensure safety and regulatory compliance. Another drawback is the scalability and cost associated with the production of hydrogel. Many hydrogels utilized in research settings are expensive and challenging to produce at the necessary scale for commercial cultured meat production, which may hinder their feasibility in the cultured meat industry[10]. Finally, some hydrogels may have limited capacity to support the intricate exchange of nutrients required for the growth of tissues on a large scale, which can hinder the development of thicker and more complex meat structures.

Recent studies have highlighted hydrogels used to create a 3D environment similar to that of the ECM[48], as a filler of 3D ECM within porous scaffolds[18] as components of bioinks[7] as thin membranes that can be micro-structured to produce cell alignment[56], or as source material to develop porous scaffolds[55]. Guzelgulgen et al.[48], used glucuronoxylan-based quince seed to fabricate a 3D hydrogel similar to the ECM. They created a porous and interconnected structure and tested cell culture and viability with NIH/3T3 cells. The ECM analysis occurred in inter/intracellular components for two months. Cell culture samples were evaluated via SEM and immunostaining methodologies; they observed that the spheroids were homogeneously scattered inside the quince seed hydrogel, where the average spheroid diameter was around 300 μm. Nuclear DAPI staining was done to investigate cellular units inside the spheroid structure. The results also confirm the homogeneous distribution of cells inside the spheroids, and ECM formation was confirmed via collagen secretion analysis. Therefore, these results prove that quince seed hydrogel is a novel scaffold material with suitable mechanical features, remarkable swelling capacity, and good biocompatibility[48]. The use of 3D ECM as a

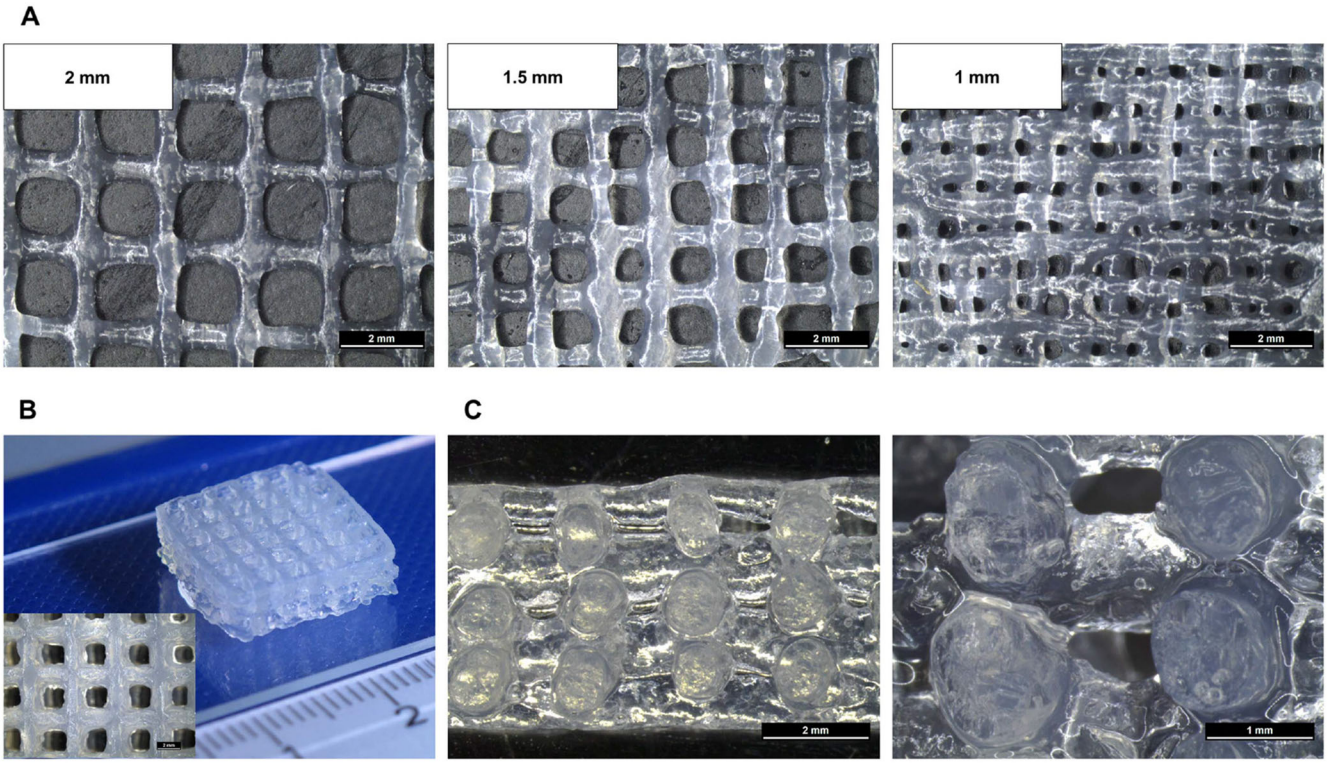

Fig. 4 | Plotted ALG/AGA/MC scaffolds after crosslinking and incubation in MS medium for 24 h. A Scaffolds with different set strand distances, two layers. **B** Scaffold fabricated in 0°/90° configuration, 20 layers, strand distance: 2.5 mm. **C** Horizontal pores in a scaffold fabricated in 0°0°/90°90° configuration, 14 layers, strand distance 2 mm (side view with enlarged section). Reprinted with permission from ref. 12, Copyright 2024 Elsevier.

filler is described by Chen et al.[18]. The authors fabricated an AG/PAAM/chitosan/gelatin scaffold and tested the growth of MC3T3-E1 cells, testing the proliferation and differentiation. The filling of PAAM reduced the pores and thickened the pore walls of composite hydrogel scaffolds, which gave rise to the enhancement of stress support and stress transfer ability, thereby enhancing the mechanical properties of AG/PAAM/chitosan/gelatin composite hydrogel scaffolds[18]. Park et al.[12] used hydrogels as components of bioinks, creating a CBF with carrot tissues. The callus-based hydrogel showed a fully opened porous construction with macropores, essential for supplying oxygen and nutrients to the cells[57]. Due to this porous structure, prolonged cell proliferation was observed during the overall incubation period, demonstrating that the cells could be successfully cultured in the hydrogel[7].

Synthetic hydrogels are commonly used for tissue engineering due to their inert biological properties, which prevent an immune response[58]. On the other hand, composite hydrogels can better mimic the ECM and show improved properties compared to those composed of a single material[22]. Hydrogels based on a blend consisting of AG/AGA/MC using EBB plotting of a basil cell-laden hydrogel were investigated for their potential use in food bioprinting applications[14]. The blend was prepared and plotted with the Bioscaffolder 3.1 (GeSiM mbH, Radeberg, Germany). After that, an in vitro cell culture of basil (*Ocimum basilicum* L.var.*purpurascens Benth*. 'Cinnamon Basil') was used for plant cell bioprinting. They observed that the mixture of the basal cell agglomerates into the ALG/AGA/MC blend did not disturb the extrusion of homogeneous strands or the fabrication of stable scaffolds. During the EBB process, it was observed that the minimum inner needle size should be 610 µm, otherwise, smaller needle sizes blocked the process. Most of the cells survived the process of 3D plotting and cross-linking, and the size and shape of cell agglomerates were similar to those in suspension. The detection of living cells at later time points of cultivation until day 20 revealed that the cells could be cultivated within the plotted hydrogel matrix. Future studies can evaluate the same approach for cultivating animal cells[12]. Figure 4 shows the plotted ALG/AGA/MC scaffolds after cross-linking.

EBB is the most used 3D printing method because it is a versatile, simple, and low-cost[12] method in which the bioink is released by a computer-controlled robotic system, resulting in the precise and continuous deposition of cylindrical filaments[58]. The limiting factor of this method is the slow printing time and the lower return of cell viability compared to the other methods, which are between 40% and 86%[58]. EBB can be done with different techniques[58]. The extruded gel often spreads after deposition, requiring an adhesion method to ensure the printed structure's stability. These adhesion methods can be photopolymerization or immersion of the printed material in a crosslinking agent solution[58]. The EBB technique was used to obtain HAP porous scaffolds[52].

Although HAP microspheres possess great bioactivity, biocompatibility, biodegradability, absorbability, and compressive properties, the high brittleness and low toughness of pure porous HAP materials have limited their practical applications. Fortunately, combining HAP nanoparticles as fillers and ductile polymer as the matrix for preparing nanoparticle/polymer composite porous scaffolds provides a promising way to overcome the shortcomings of pure porous HAP materials[59]. PCL is another polymer that has received much attention owing to its favorable biocompatibility, biodegradability, and processability[60,61].

In the 3D inkjet printing method, small drops of liquid ink are produced and deposited on the substrate[62]. This method is considered versatile, affordable, accurate, and achieves good resolution, which returns cell viability between 70% and 96%[62]. Still, there are limitations on its use, especially concerning the bioink used, which must have low viscosity and a cross-linking mechanism to stabilize the printed structure[63]. Another limitation is the small nozzle, which leads to clogging and impairs cleaning, which makes production difficult and reduces efficiency in large-scale manufacturing. This method is based on printing multiple and not continuous drops, and this lack of mechanical integrity makes it not favorable to its use for printing[47]. On the other hand, Chen et al.[64] demonstrated that the

production of scaffolds by PLGA created an environment that provides the appropriate conditions for the growth, differentiation, and survival of C2C12 myoblasts, simulating the complex structure of the ECM. The results showed that in 3D printed scaffolds, the survival rate was higher than in the control made in films and PLGA spheres[63]. In addition, the scaffold with a 50 μm fibrillar gap was the most suitable because it demonstrated increased cell adhesion and proliferation compared to the others. Thus, it was observed that these scaffolds have a controlled and uniform architecture, proving that 3D inkjet printing is a suitable tool for manufacturing cell culture scaffolds with defined structures[63].

LAB is based on the deposition of liquid bioink on a metal-coated surface, followed by laser-induced cavitation on the tape, which forms micro bioink droplets. LAB preserves cell viability by more than 95% and is compatible with bioinks with a large viscosity range (1–300 mPa/s), and because their deposition is done without a nozzle, clogging is not a problem for this method. Using LAB combined with other methods is another possibility. Nawroth et al.[65] applied ultraviolet laser-activated photosensitizer (UVL) to create hydrogel patterns. This technique returned a short manufacturing time and high standardization volume compared to conventional methods[65].

Another laser-assisted manufacturing technique is laser-induced forward transfer (LIFT) bioprinting[50]. This method comprises an upper layer designed for energy absorption, a middle layer acting as the donor, and a lower layer consisting of the bioink. LIFT entails the vaporization of the donor layer upon exposure to a laser beam directed at predetermined points, inducing the generation of high-pressure bubbles at the interface. This pressure triggers the bioink transfer to the collection phase, culminating in a three-dimensional model creation[50]. The advantages of using LIFT include the high rate of cell viability and utilization of highly viscous materials[66]. One of the drawbacks is the laser cost and its difficulty in control, besides the metallic residue in the final product, which is a concern regarding the final product safety, restricting the use of LAB for the production and marketing of CM.

Stereolithography uses a matrix of digital micromirrors to adjust the intensity of the visible or UV light beam, curing photosensitive polymers layer by layer[51]. This method is fast, inexpensive, and returns cell viability above 85%[67]. One of the limitations of its use is that light-blocking agents are used for photoresist standardization and are not suitable for food applications because they are toxic and carcinogenic[67]. On the other hand, callus-based food inks (CBF) were formulated for stereolithography[7]. Ratios of CBF to AG were tested at 1:2, 1:1, and 2:1 (w/w). Shear-thinning behavior was observed across all scaffolds, indicating a decrease in viscosity as shear rates increased, a critical property for effective 3D printing. This shear-thinning characteristic allows CBF to flow through a fine nozzle at high speeds during the printing process[68]. The 1:2 and 1:1 CBF formulations displayed fine resolution regarding layer width and pore diameter, resulting in well-defined printed structures with dimensions close to their intended targets. However, interlayer adhesion became apparent at higher cell concentrations, such as in the 2:1 CBF sample, possibly due to its lower alginate content and higher cell density. In cell-laden ink systems used for 3D printing, an increase in cell concentration typically results in more cells aggregating at the liquid–liquid interface, thus reducing the surface tension and total free energy[57]. This reduced surface tension causes the CBF to flow quickly through the nozzle during the printing process and spread, leading to issues with interlayer adhesion. The curing test results proved this observation, showing that the 2:1 CBF sample, which had lower alginate content, did not bind sufficiently to Ca^{2+} in the gelatin slurry, leading to inadequate gel strength. This finding suggests that cell concentration is crucial to improve printing accuracy and maintain structural integrity during incubation. Cell growth within the CBF lattice scaffold was assessed by culturing for 35 days. The printed lattice scaffold had a fully open porous structure with macropores, providing the necessary pathways for oxygen and nutrient delivery to the cells[69]. This porous architecture enabled sustained cell proliferation throughout the incubation period, indicating that the cells could successfully grow in the CBF gel[10].

Electrospinning

Electrospinning is a simple, inexpensive method already used in various industrial branches, such as the textile industry, nanotechnology, tissue engineering, and cellular agriculture[70]. The products generated by electrospinning can be made on an industrial scale. However, although it is versatile and has the potential for large-scale production, its application in food systems has not yet been fully elucidated[2].

The electrospinning process uses electrostatic force to stretch droplets of a polymer in solution to their potential point, forming a structure called a Taylor cone[71]. Upon reaching a critical value above the droplet surface stress, a polymer solution jet is released, diluting simultaneously as the solvent evaporates, forming submicrometric or nanometric solid fibers constantly deposited in a grounded collector[71]. The filaments produced vary in size and microstructure to fit the desired application. This adjustment can be made depending on the polymer, chosen solvents, environmental factors (temperature, humidity), and process parameters[72].

Fibrous scaffolds are made through electrospinning, which can produce nanofibers with various useful properties for CM. Some of these properties are the ability to support cell adhesion, perform the diffusion of oxygen and nutrients, and produce aligned fibers that promote muscle fiber maturation[57]. Polymeric materials for spinning techniques include PCL[72] and cellulose acetate (CA)[12], as shown in Table 1. Also, common material combinations that can improve scaffold properties, such as PVP + PGS[21] and melanin+PHB[33], are also shown. Plant/fungi-based material by electrospinning, such as fungal mycelial mats with chitin-glucan polysaccharide cell walls[29] and CA + SPH[8] are promising.

Even though it is a promising technology aimed at large-scale production, producing scaffolds for CM using electrospinning has some challenges that need to be overcome. One of the main challenges is the need to use non-edible solvents, such as fluorine-alcohols, hexafluoro isopropanol and 2,2,2-trifluoroethanol, which denature proteins and provide elastic, viscous properties, allowing fiber formation during electrospinning[73,74]. These solvents have a high evaporation rate and partially denature biopolymers made by electrospinning, breaking hydrophobic interactions, and hydrogen bonds[2]. They are considered toxic and unsafe for food because they can leave residue even if they are quickly evaporated. As an alternative for the electrospinning of edible biopolymers, high ionic force aqueous solutions or benign solvents, such as ethanol[75], formic acid, or acetic acid[76,77] can be used, which are classified as Class 3 solvents by the Food and Drug Administration (FDA). Also, they are less toxic, have lower risks to humans, and can be included in food in restricted quantities by good manufacturing practices.

Another solution is the addition of carrier polymers, which increase the spinning capacity of the electrospinning solution by improving its viscoelastic properties[76]. They must be degradable in the human digestive system, and the concentration of the polymer and its degradation products should be atoxic, as determined by authorities such as the FDA. Poly (ethylene oxide) (PEO)[65], PCL[50], and PLA[66] are used as carrier polymers in the electrospinning of edible biomaterials. Among these polymers, only PEO is approved by the FDA as an indirect food additive. In addition, these synthetic polymers do not provide nutritional benefits or support cellular adhesion, which is essential for cellular scaffolds. Therefore, these polymers are undesirable for direct consumption, and alternatives should be investigated to develop CM[67] scaffolds.

Ahn et al.[8] used CA as a carrier polymer, which increases fibroblasts' proliferation, growth, migration, and infiltration. Using jet electrospinning, they built a plant-based scaffold made from hydrolyzed SPH and CA. The RJS system's polymer concentrations significantly influenced the spinnability and beading of CA and SPH nanofibers (w/v%). The SPH has bioactive peptides similar to the proteins that make up the ECM, which promote cell adhesion, proliferation, and migration to tissue regeneration[78], but SPH itself could not be spun into nanofibers because its molecular weight is too low. The short chains of SPH molecules cannot overlap and entangle, suggesting that SPH would require a co-spinning polymer with longer chains[79]. Ten w/v% of CA was therefore selected as the carrier

polymer for SPH. The developed continuous nanofibers had an intercalated structure that resembled the native ECM. The composite scaffold showed lower cytotoxicity when compared to nanofibers made of PCL or only CA[8].

To overcome the solvent problem, Narayanan et al.[29] used β-mercaptoethanol (BME) and observed improvements in hemocompatibility and biocompatibility once BME conferred scaffolds made of fungus adhesion and proliferation of keratinocytes. BME is toxic in high concentrations. In cell culture, it is often used in very low concentrations (e.g., 0.1 mM or less) to minimize toxicity while still maintaining its reducing properties. Even at low concentrations, residual BME could pose health risks if it remains in the final product. Therefore, it would be advisable to remove or neutralize BME before the cells are harvested for meat production and implement careful control measures to ensure it does not remain in the final product. Further investigation must be done on the applicability of CM scaffolds. The authors demonstrated the manufacture of a cross-linked scaffold of chitin-glucan polysaccharides made of fungi using electrospinning. Fungi are a group of eukaryotes; they have cell walls composed of chitin, are highly branched from hyphae, and grow as rigid structures, very similar to micro and nanofibers made by electrospinning[80]. Still, on the cell walls of filamentous fungi, they are constituted by several linear structures and branched polysaccharides, as well as proteins modified after translation and lipids. This mycelial organization in filaments offers mechanical resistance and promotes interactions with the host elements[57], justified by using these biomaterials to construct scaffolds.

For application in scaffolds, the nanofibrous pores mimic the morphology and structure of ECM tissues and have a large surface area, making them ideal for adhesion and proliferation[62]. The orientation of the electrospun fibers can be adjusted to control the morphology of cells grown on the scaffold. For example, electrospun fibers can be aligned, which induces the alignment of seeded cells and promotes the stretching of muscle cells andyogenesis[81]. Cell-loaded polymer solutions can be electrospun, and the micro-pattern of the electrospun filaments can guide cell growth, resulting in homogeneous cell distribution and greater accessibility of nutrients throughout the scaffold[82].

In addition, electrospun blankets may undergo post-processing modifications, such as chemical or physical crosslinking, to improve their mechanical properties. Some of the protein crosslinks usually used are considered toxic, including formaldehyde[83]. Therefore, these crosslinkers should be avoided in food applications. Non-toxic crosslinkers, crosslinking enzymes, or physical crosslinking modes using pH or temperature can be used instead to obtain scaffolds of superior mechanical properties[81].

Microcarriers

Microcarriers are made from the growth of adherent cells in small suspended particles[84]. The microcarriers are mainly made of PE, crosslinked dextran, cellulose, gelatin, or polygalacturonic acid (PGA), coated with collagen or peptides containing adhesion or positive charges to promote cell adhesion. The diameter of a microcarrier is between 100 and 200 µm[25]. Bodiou et al.[25] describe existing microcarrier production technologies and how they can be adapted as CM scaffolds. Three possibilities for using microcarriers were raised. The first would be as a temporary carrier aimed at supporting cell proliferation and being removed before processing. Secondly, the temporary carrier is dissolved or degraded to release the cells. Finally, the microcarriers are an edible scaffold incorporated into the final product. Examples in Table 1 include the temporary carrier being dissolved or degraded approach[85] and microcarriers as an edible scaffold incorporated into the final product[9,17]. It is common to use microcarriers to scale cell proliferation in bioreactors, as they provide anchorage for suspended cells[39]. For this reason, decellularized plant-based microcarriers can serve as a key factor in scale-grown and affordable meat production[39]. Although microcarriers offer a relatively simple solution to expand mammalian cells on a large scale and require little space, they have limitations regarding cell dissociation and separation costs, the cost of the microcarriers themselves, the maximum cell densities that can be achieved, and potential impacts on the nutritional and sensorial properties of the final product[22].

One of the techniques that can be explored in microcarriers is microfluidic[17] (Fig. 3). Microfluidic technology is dedicated to studying and manipulating all fluid volumes in miniature systems, using channels with dimensions between 10^{-9} and 10^{-8} l[86]. These channels combine chemical compounds to synthesize and separate substances through a pumping technique. Unlike macro scales, where physical characteristics and mass transfer based on diffusion are linearly scalable, these properties cannot be extrapolated directly at the microscale. The main advantage of the microfluidic technique is obtaining a laminar flow, which is an impossible phenomenon to achieve in large-scale devices due to the predominance of viscous forces[86].

Due to their suitable physicochemical characteristics, monocrystalline silicon and borosilicate glass are commonly used to build microfluidic platforms. In addition, polymers have been widely used in manufacturing these devices, with polydimethylsiloxane (PDMS) being one of the most favorites[87]. The PDMS can be easily shaped into channels with high accuracy in terms of micrometer size, transparency to light, and low water permeability. However, an important disadvantage of PDMS is its lack of resistance to organic solvents, such as amines, strong acids, and hydrocarbons, which led to the development of solvent-resistant microfluidic reactors[87,88].

To achieve better adhesion of the cells, the microcarrier should have a porous surface[89]. Porous scaffolds have a sponge-like structure (Fig. 3), with a pore size in the micrometer range[90]. This structure provides the mechanical stability necessary for cultured cells to form tissues. These scaffolds resemble the structure, mechanical properties, and composition of the connective tissue of the perimysium[55], considering that the scaffold would remain a component of mature tissue. Commonly used porous scaffold manufacturing techniques such as particle leaching, melt molding, freeze-drying, and gas foaming[91], usually use synthetic polymers, which must be replaced by edible structure[91] for CM application. Pore size, porosity, and scaffold material are key factors affecting tissue development and cell survival. While pore size is important for cell culture, the integration of larger pores suitable for medium perfusion should also be considered for pseudo vascularization[92] to enable the efficient transport of nutrients and oxygen in thicker scaffolds for CM.

Kankala et al.[93] were motivated by the lack of research on the applicability of porous microcarriers, and, as a result, they manufactured microspheres with highly open pores using a microfluidic technique. These microspheres were designed to house skeletal structures in myoblast proliferation and were subsequently evaluated for their viability in cell delivery. The biocompatible microspheres produced had particle sizes between 280 and 370 µm and pores with dimensions between 10 and 80 µm. This structure provides a favorable microenvironment, allowing cells to be closely arranged in elongated forms with the deposited ECM, facilitating adhesion, proliferation, and increased myogenic differentiation of cells. Using PLGA to manufacture porous microspheres allowed a minimally invasive cell delivery system creation. The study demonstrated a high cell adhesion rate, continuous proliferation, and increased myogenic differentiation of C2C12 when organized in fibrous layers in porous microspheres. Additionally, the porous microspheres presented an established ECM and exhibited a strong potential for myoblast differentiation, which facilitated the growth of these skeletal muscle cells concomitantly with vascularization[93].

Lyophilization is another technique that can be used in microcarriers. It consists of a drying process in which a solvent, usually water, is removed from a product by sublimation[94]. This process has already been used to manufacture porous scaffolds for cellular agriculture[94]. The lyophilization process can be divided into three stages: solidification, primary, and secondary. In the first phase, solidification, the solution begins to be cooled to a temperature below its eutectic point, which is the point at which the entire sample is frozen. Subsequently, the vacuum is applied in the second phase to reduce the pressure and facilitate the sublimation process. The process transitions from solid to steam, beginning in the first phase. During the first drying, unbound water is removed from the material, leaving only a porous structure. In the third phase, the secondary drying, the sample is heated,

facilitating the unbound water desorption[95]. Still, in the freeze-drying stages, it is known that the primary drying is the slowest one[95]. If the established time is inadequate in the primary drying, the removed solvent will be insufficient, and what remains in the sample will be heated during secondary drying, spoiling the sample. Temperature is also a determining factor in primary drying. Both temperature and time influence the crystal size formed, affecting the pore structure[95]. The primary drying phase presents additional opportunities to control the physical properties of the scaffold by monitoring the interactions between the sample and the bound water. Previous studies have shown that manipulating these interactions, changing drying rates, and time considerably affect scaffold stiffness and secondary structure formation[96,97].

Although drying techniques like freeze-drying and spray-drying are well-established and optimized in pharmaceutical applications, their application in scaffold manufacturing is still emerging. Unique challenges, including variations in material composition, porosity, and mechanical stability, complicate the direct adaptation of these methods, highlighting the need for further research in this area. In their study, Abbott et al.[98] demonstrated the influence of time and temperature on primary lyophilization drying to produce porous scaffolds. Four different solutions were tested, varying the concentration of water/volume of solution in 3%, 6%, 9%, and 12%, using three distinct protocols: long hold, slow ramp, and standard. The long hold and slow ramp protocols resulted in scaffolds from all concentrations, while the standard did not work well for 9% and 12% concentrations. In order to investigate the use of different scaffolds, a live cell of the HepaRG line was grown on scaffolds of all concentrations made by the Long Hold protocol. Initially, the scaffolds of each concentration showed variations in lipid accumulation, cell growth, and metabolic activity, but these differences were no longer observed after the 28th day of culture. It was possible to conclude that by modifying the parameters of the primary drying and the concentration of the solutions, it is possible to obtain lyophilized scaffolds with suitable properties for cellular agriculture[91].

Enrione et al.[94] also used the lyophilization technique to produce porous scaffolds. Four polymeric solutions were created: all containing salmon gelatin and sodium alginate, two with agar, two with agarose, and one in each of these two groups with glycerol. The concentrations of each component were not varied. These scaffolds were tested for cell line cultivation of myoblasts C2C12. The most promising scaffold contained salmon gelatin, sodium alginate, agarose, and glycerol. The pore size obtained for this scaffold was around 200 µm in diameter, the biocompatibility and adhesion of myoblast cells were around 40%, and it took around 24 h to double the growth rate[80]. The biodegradation profile of scaffolds was lower than 25% after 4 weeks; they also had adequate myogenic response, high cell proliferation and viability, and adequate cell distribution[80].

Decellularization

Decellularized structures derived from plants or fungi can also produce scaffolds. It can provide natural 3D structures, which facilitate the transport of oxygen and nutrients essential for cell growth[99,100]. Using these natural structures as scaffolds can reduce the complexity of the manufacturing process and increase the efficiency of CM production[39]. The process of tissue decellularization can be carried out by physical, chemical, and enzymatic methods[39]. The most commonly used physical methods are faster freezing or freezing-thawing. In freezing, intracellular ice crystals are formed in tissues, disrupting cell membranes and triggering cell lysis[101,102]. In freezing-thawing, there must be precise control over temperature because it affects the size of the ice crystals formed, a factor that can degrade the ECM.

The lack of native cell adhesion molecules (fibronectin, integrins, and collagen, which are natural in animal tissues and crucial for cell attachment and signaling in animal cells) in plant-derived scaffolds and the biochemical incompatibility with animal cells may hinder cell attachment and growth. The limitations of those scaffolds include their mechanical stiffness and the absence of essential cell adhesion sites, which makes it difficult for animal cells to recognize and adhere to plant-based surfaces[103]. Therefore, surface modification is necessary because plant-based scaffolds do not inherently possess the biochemical and physical properties required to support the attachment and growth of animal cells. By modifying the surface, it is possible to create a more favorable environment for cell adhesion, thereby improving the functionality of plant scaffolds in CM applications[104]. Thus, to overcome these limitations, it is possible, for instance, to introduce biochemical cues and topographical features (micro- and nano-patterns), in order to improve cell attachment, proliferation, and differentiation, besides functionality on scaffolds[83].

Chemical decellularization typically employs detergents, acid/alkaline solutions, and chelating agents[105]. In a study on chemical decellularization, researchers explored the use of decellularized apples coated with alginate/gelatin as a bioscaffold for CM production. They created two types of 3D scaffolds, uncoated (A) and coated (CA), varying pore size distributions ranging from 100 to 250 µm. The decellularization process involved treating thinly sliced apples with SDS, washing them, and then placing them in a beaker with SDS, maintaining the solution at 25 °C with 150 rpm agitation for five days. After decellularization, the scaffolds were crosslinked with a gelatin and alginate polymer blend and then freeze-dried. This crosslinked polymer coating increases the surface area for cellular metabolic activity and can contribute to the scaffold's meaty texture. Satellite muscle cells were seeded onto scaffolds to test cell support[106], and coculture of NIH/3T3 cells and muscle satellite cells was established to assess cell growth. Co-culturing these two cell types was successful on both scaffolds, but it was more pronounced on the CA scaffolds, likely due to the polymer coatings enhancing cell adhesion (Fig. 5). The coculture remained viable for seven days, indicating that both muscle cells and NIH/3T3 fibroblast cells could sustain growth in this medium[107].

Enzymatic decellularization offers some advantages, such as reducing cellular residues, but removing enzymes after the completion of the process is difficult, imposing limitations on the utility of enzymatic treatments[108]. Enzymes used for decellularization include trypsin and pepsin[109]. In a recent investigation by Thyden et al.[47] rapid decellularization of broccoli was achieved, demonstrating the capacity of decellularized broccoli to support the adhesion and viability of BSCs within a dynamic reactor environment. Furthermore, decellularized broccoli exhibits physical and nutritional attributes that can offer advantages in both the production and consumption of CM[85].

Studies have investigated the use of spinach for scaffolds based on its wide availability, dense vascularization, and wide petiole (stem that connects the leaf to the stem)[99,100]. Another example of a vegetable that has been explored is apple[110,111]. The authors demonstrated that decellularized apples can support the adhesion and survival of C2C12 myoblasts during a two-week culture period and promote binding and proliferation of induced pluripotent stem cells (iPSCs) differentiation in bone tissue[110]. These characteristics make plants an attractive and sustainable alternative for the manufacture of scaffolds.

Types of scaffolds in bioreactors

The dynamic culture in bioreactors involves the creation of a controlled environment in which microorganisms, cells, or organisms are grown in conditions that mimic the natural fluctuations that occur in their native environment. Dynamic culture is essential for CM production, as it more closely simulates the natural conditions of cell growth, leading to healthier cells and more efficient production of desired compounds. This method involves varying parameters, such as agitation, aeration, temperature, and nutrient concentration over time, in response to the needs of cultured cells.

Agitation bioreactors emerge as a highly effective alternative for scaling up stem cell production[112]. The dynamic cultivation within these bioreactors provides a hydrodynamic environment that favors fluid movement throughout the system, improving the transport of nutrients and the mechanical stimuli that drive the production of the ECM. In addition, it is essential to carefully consider the density and the method of cell sowing since an adequate number of cells is crucial to ensure appropriate cell interactions, homogeneous distribution, and penetration into the structural supports[112]. Several types of bioreactors allow the application of unconfined

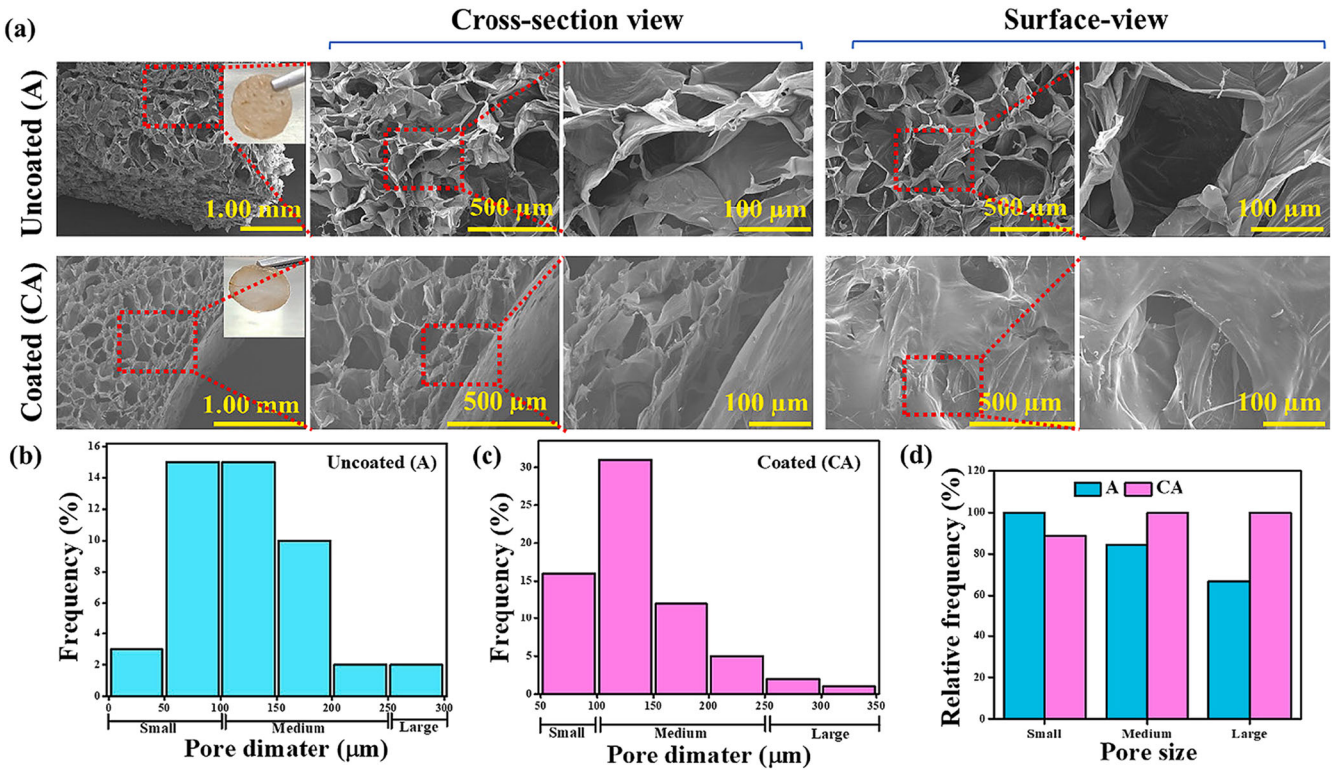

Fig. 5 | Morphological and structural characterization of decellularized apple scaffolds. a Scanning electron microscopy (SEM) images of cross-sectional (left) and surface (right) views of uncoated (A) and coated (CA) decellularized apple scaffolds. **b** Pore size distribution analysis of uncoated scaffolds (A) and **c** coated scaffolds (CA). **d** Comparative histogram illustrating the relative frequency of pore sizes in uncoated (A) and coated (CA) scaffolds, highlighting differences in porosity profiles. Symbols: A = uncoated scaffold; CA = coated scaffold. Reprinted with permission from ref. [107], Copyright 2024 Elsevier.

compressive forces and shear. Models such as the balloon bioreactor with mechanical agitation and the rotary wall have been designed to apply different shear intensities to the cell support structures[112]. The fluid circulation within these configurations generates shear forces, which, as reported by Darling and Athanasiou[113] promote the production of the ECM. The rotary flask is the most common and simple among the mechanical stirring approaches. This device effectively mixes oxygen and nutrients throughout the medium, reducing the formation of concentration gradients on the surface of the support structures[113].

The fluidized bed bioreactor is a perfusion system characterized by the continuous upward flow of fluid around immobilized particle cells, allowing them to behave like a fluid. This type of reactor has attracted increasing interest within tissue engineering due to its ability to expand the bed homogeneously, its excellent mass transfer properties, and its adaptability for large-scale applications[114]. One of the advantages of the fluidized bed bioreactor is the continuous perfusion of the medium, eliminating the typical feeding cycles of static and discontinuous cell cultures. This approach theoretically allows for achieving higher cell densities, ensuring a constant replenishment of nutrients. However, it is important to consider that increased turbulence to improve mass transfer may also result in cell damage and mortality due to the resulting shear forces[115]. Differing from tank reactors with continuous stirring and bag reactors, commonly employed in the industry for large-scale cell cultures, fluidized bed bioreactors are particularly efficient in cultivating cells sensitive to shear stress, such as mammalian cells[116]. Effective mixing of the medium without needing mechanical agitation, impellers, or aeration represents a valuable feature that simplifies bioreactor construction and reduces maintenance and operating costs. Although 2D cultures are still performed in static flasks or plates in laboratories, large-scale production based on flat surfaces is inefficient. Gel granule encapsulation, using a suitable matrix or seeding in 3D microcarriers, has been widely adopted to overcome the surface limitation for adherent cells. This approach provides a greater proportion of area/volume for cells to settle and perform mass transfer while reducing the space required for cell cultivation in bioreactors.

Additionally, the encapsulation provides a protective layer for the cells. The resistance of gel granules is a critical feature and can be adjusted to minimize the rupture or loss of matrix cells. These gel spheres must be sufficiently robust to withstand abrasion and compression generated by contact between particles[117]. When operating the bioreactor under fluidization conditions, it is essential to apply a surface velocity higher than the minimum fluidization velocity, where the particle bed begins to expand but is lower than the terminal velocity at which the particles are expelled from the system. It is essential to characterize the particle bed expansion behavior to ensure the efficiency of the fluidized bed bioreactor design in cellular agriculture applications[92].

Scaffold-free approaches

Although scaffolds provide many advantages for cellular agriculture, such as facilitating the transport of oxygen and nutrients and precise control over the 3D geometry of the final construction, scaffold-free methods can also solve these challenges[150]. Combining several planar cells is one of the methods that can be used to manufacture scaffold-free CM. In this case, as bioinks and scaffolds are not used, the cells are held together by their secreted ECM. One of the employed techniques is cell sheet technology, which involves the preparation of cell sheets within temperature-responsive culture dishes (TRCDs)[118]. These TRCDs feature a surface covalently bonded with a temperature-sensitive polymer, Poly (N-isopropyl acrylamide) (PIPAAm), through electron beam irradiation. This bonding renders the surface hydrophilic below 32 °C and hydrophobic at 37 °C[119], facilitating cell adhesion at the lower temperature and maintaining cell-to-cell cohesion at 37 °C. To detach the cell sheets from the dish surface while preserving their structure, a temperature below 32 °C was employed, producing cell sheets. By stacking and attaching multiple cell sheets, 3D constructs with a thickness of several millimeters can be readily fabricated, characterized by high

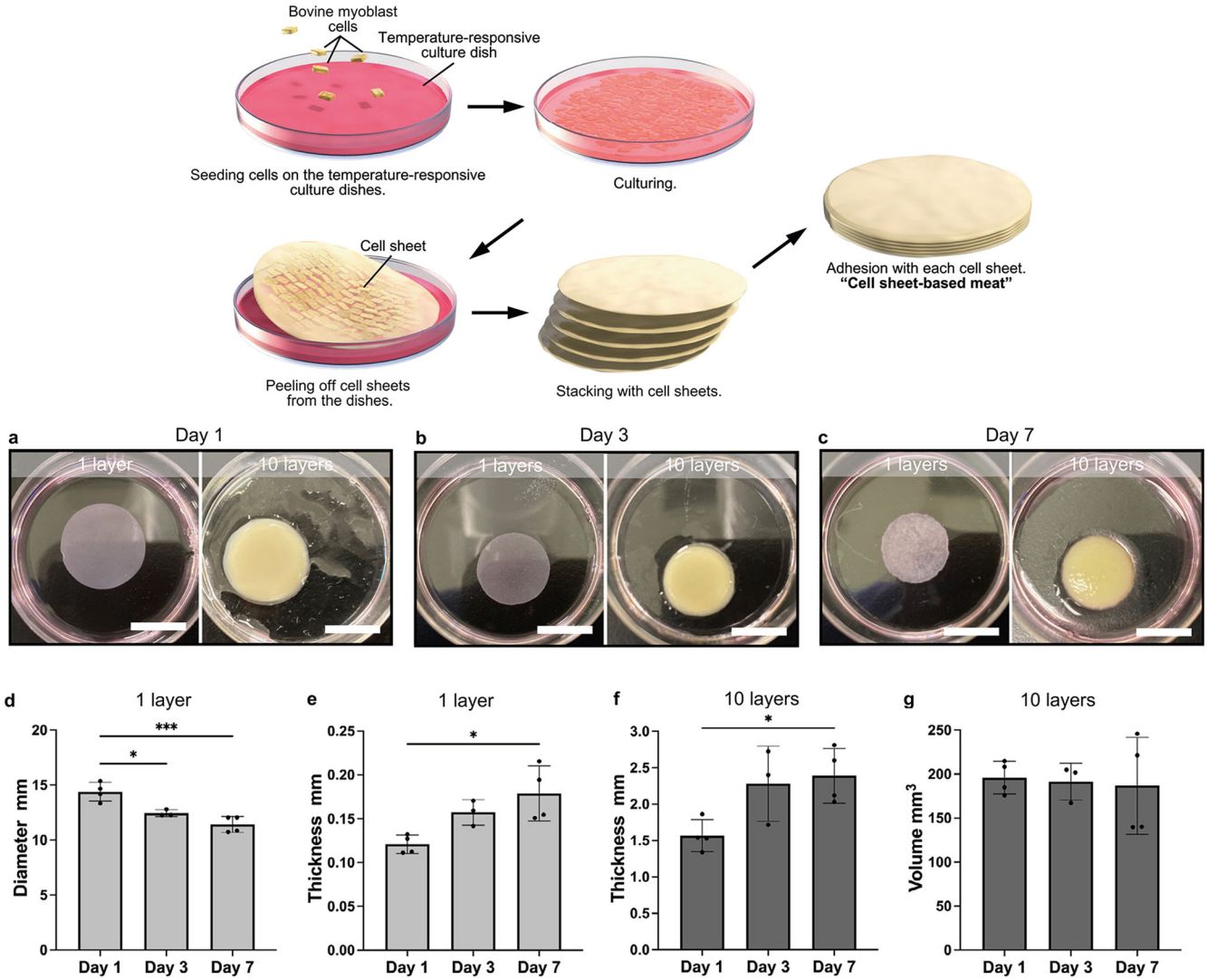

Fig. 6 | Production of cell sheet-based meat. Dimensional and structural characteristics of the bovine myoblast cell sheets. **a–c** One and 10-layered-bovine myoblast cell sheets. **a** Day 1 of culture. **b** Day 3 of culture. **c** Day 7 of culture. Scale bar: 1 cm. **d** Diameter and **e** thickness of the individual bovine myoblast cell sheets ($n = 4$). **f** Thickness and **g** volume of the 10-layered-bovine myoblast cell sheets ($n = 4$). Reproduced with permission from ref. 120. Copyright 2024 Elsevier.

cell density. This approach generated various tissue types, such as skeletal muscle[112], liver tissue[113], and cardiac tissue[114] in vitro. Due to its scalability, this method is also suitable for CM production. Tanaka et al.[120] utilized TRCDs and stacked 10-cell sheets to produce three-dimensional tissue with a thickness ranging from 1.3 to 2.7 mm using bovine myoblast cells. Results indicated an increase in hardness after incubation in TRCDs, with further enhancement observed post-boiling, mimicking the texture of natural meat. The cell sheet exhibited approximately half the wet weight percentage of total protein compared to beef. The scheme in Fig. 6 shows how the method works. The primary challenge with stacking cell sheets is ensuring that all cells within the layers receive adequate oxygen and nutrients. Without a vascular network, diffusion is limited to about 200 micrometers from the nearest capillary, which restricts the thickness of viable tissue. As the number of layers increases, cells in the inner layers may suffer from hypoxia and nutrient deprivation, leading to cell death and reduced tissue functionality. Up to 10–20 layers of cell sheets have been successfully stacked under laboratory conditions, but beyond this, the limitations of oxygen and nutrient diffusion become more significant without additional interventions.

Based on a pH change, another study[69] used the π-SACS method to trigger delamination of a cell sheet, which is later stacked with other sheets, to form C2C12 myoblast cells. This method was also recently explored by Shahin-Shamsabadi and Selvaganapathy[121] for use in CM from a combination of muscle cells and adipocytes. The challenges of the π-SACS method include the space required for cell growth in 2D culture and the laborious nature of stacking multiple sheets. In order to address these issues, new bioreactor geometries and automated methods for fabric assembly should be studied[30].

Final considerations and future prospects

It is widely known that the fibrous texture of meat results from its hierarchical tissue structure, in which muscle fibers form the primary functional units. These muscle fibers, along with intramuscular fat, vasculature, and nerve structures, contribute to the overall composition of meat. Structurally, muscle fibers are arranged in bundles that are encased by connective tissue[23]. This connective tissue is organized into layers known as the endomysium, perimysium, and epimysium, which surround individual muscle fibers, fiber bundles, and entire muscles[23]. Although vascularization does not directly contribute to the sensory properties of meat, such as taste and texture, it is essential for transporting oxygen, nutrients, and minerals throughout the tissue[53]. For simple products like ground meat, the need for vascularization may be less critical, as smaller cell aggregates or thin sheets can be cultivated and then combined. However, for structured meats, such as steaks or other complex cuts, which require a certain texture and thickness, vascularization

is necessary to mimic natural tissue structures[122]. Similarly, while replicating nerve structures is not required to achieve the desired sensory properties in CM, nerves can aid in the maturation of muscle fibers, enhancing the quality of the final product[100].

To achieve viability in CM production and commercialization, it is imperative to utilize safe, sustainable raw materials that can be produced on a large scale. The first CM hamburger, weighing 85 g and developed by Professor Mark Post in 2013, cost ~200,000 dollars to produce. By 2019, the price had dropped significantly, with the same burger costing around nine euros. Ensuring food safety is paramount, and materials must adhere to regulations set by authorities such as the FDA[17]. Furthermore, sustainability in CM production requires the use of inputs that minimize environmental impact. Animal raw materials such as fetal bovine serum are used for studies and tests but should not be applied to manufacturing food grown on a large scale[10]. The chosen biomaterial must support cell culture and possess mechanical and biochemical structures to guide the connection between cells, their morphology, proliferation, and other cellular activities[17]. The biomaterial must also support cell differentiation of meat-related cells such as myocytes, adipocytes, or fibroblasts[123]. The compatibility of a certain biomaterial with the desired manufacturing technology dictates its technological feasibility.

Biomaterials that may be allergenic, such as soy and peanuts, should be properly specified on the label. Some suggested biomaterials for scaffolds are proteins such as collagen, gelatin fibrinogen, and polysaccharides[10]. Collagen, gelatin, and fibrinogen are naturally abundant in the ECM of animal tissues, offer good cell adhesion and growth, and add nutritional value. However, because they are of animal origin, these proteins are less acceptable in terms of sustainability and animal welfare. One way around this problem may be new technologies that produce recombinant proteins through plants and fungi[124].

Research on extracting cell-adhesive particles from fungi for CM applications is also an emerging field. Fungal-derived biomaterials are promising scaffolds for tissue engineering due to their biocompatibility, sustainability, and inherent ability to promote cell adhesion. For example, studies have explored the adhesive properties of fungal cell walls for creating three-dimensional structures to support muscle cells, while others focus on fungal-derived polysaccharides like chitin and β-glucans as scaffolds for CM cell proliferation[106]. Specifically, Teo et al.[125] demonstrated the extraction of fungal-derived protein particles that mimic natural cell adhesion molecules, successfully applying them as matrices for cell-cultivated food. To contextualize these advances, a review by Alaneme et al.[106] synthesizes the use of fungal biomaterials in tissue engineering, emphasizing their suitability for CM production through adhesive proteins and polysaccharides.

So far, the biomaterials proposed for CM meet all or part of the above-mentioned considerations. In addition, several CM scaffolds depend on modifications of biomaterials, prospective sources of biomaterials, or future technological capabilities to facilitate such compliance. Suggested scaffold biomaterials for CM include several types of proteins and polysaccharides[22]. Proteins of vegetable origin, such as proteins isolated from soybeans, wheat, oats, cotton, peanuts, and peas, are good options due to their low cost, high nutritional value, great knowledge regarding their processing, and consumer acceptability. These proteins are considered promising biomaterials for the production of CM; however, their insufficient cellular adhesion may require modifications[6].

At present, many of the biomaterials proposed for CM production meet some, but not all, of the necessary criteria. Continuous optimization is needed to ensure that these materials are compatible with both existing and emerging biomanufacturing technologies[106]. Plant-derived proteins, such as those isolated from soybeans, wheat, and peas, represent promising options for CM scaffolds due to their low cost, high nutritional value, and general consumer acceptability. Nevertheless, their insufficient cellular adhesion may require chemical or physical modifications to enhance their functionality. Polysaccharides such as chitosan, alginate, pectin, cellulose, and starch are also regarded as promising candidates for scaffolds, as they support cell adhesion and proliferation while being cost-effective and edible[8]. Finally, the chosen biomaterials must contribute to the appearance, flavor, texture, and nutritional value of CM products, helping to ensure that they closely resemble traditional meat[55].

The continued progress in research and development related to scaffolds for CM is essential, as ongoing optimization of biomaterials and manufacturing strategies will ensure the compatibility of desired cells and facilitate the production of CM that meets consumer expectations. Advances in scaffold technology and material science will play a key role in ensuring that cultivated meat products are both sustainable and scalable, allowing for wider adoption and commercialization.

Data availability

The authors declare that all data supporting the findings of this study are presented in the article. Additional data are available on request.

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G.A.S.: Conceptualization, investigation, visualization, writing—original draft. V.F.: Investigation, writing—review, and editing. A.P.A.B.: Validation, figure edition, writing—review and editing. A.K.: Validation, writing—review, and editing. S.V.: Funding acquisition, project administration, supervision, validation, writing—review and editing. All authors read and approved the manuscript in its present form.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to Silvani Verruck.