Tissue Engineering Challenges for Cultivated Meat

Subjects: Cell & Tissue Engineering | Biotechnology & Applied Microbiology

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Cultivated meat (CM) technology has the potential to disrupt the food industry—indeed, it is already an inevitable reality. This new technology is an alternative to solve the environmental, health and ethical issues associated with the demand for meat products. The global market longs for biotechnological improvements for the CM production chain. CM, also known as cultured, cell-based, lab-grown, in vitro or clean meat, is obtained through cellular agriculture, which is based on applying tissue engineering principles. In practice, it is first necessary to choose the best cell source and type, and then to furnish the necessary nutrients, growth factors and signalling molecules via cultivation media.

Keywords: biotechnological ; animal-free medium ; 3D models

1. Introduction

Cultivated meat (CM), also known as cultured, cell-based, lab-grown, in vitro or clean meat, has gained prominence in recent years due to increasing societal and industrial interest. CM has arisen as an alternative to help solve environmental, health and ethical issues related to meat product demands [1]. Cellular agriculture is used to produce CM. Its development has been promoted by different concerns: (1) the increase in the global population; (2) the environmental impact of animal agriculture, such as land use, greenhouse gas emissions and impact on biodiversity; (3) animal ethics, including livestock living conditions and slaughter; and (4) the impact of animal agriculture on human health through issues such as animal-borne disease and antibiotic use [2].

Instead of slaughtering animals for food, the meat cultivation process usually starts by obtaining a biopsy and isolating cells. Then, these cells are cultivated to grow and differentiate into skeletal muscle cells. This process can begin with different cell types, but stem cells are an obvious choice due to their ability to proliferate and to differentiate into several lineages [3]. The cells can be obtained from two sources: adult stem cells, which have a limited proliferative capacity, or pluripotent stem cells, which have an indefinite proliferative capacity. The cells usually employed for this application include: (1) muscle satellite cells that are muscle stem cells that can differentiate into myotubes, which is the target cell type; (2) mesenchymal stem/stromal cells (MSCs) that can differentiate into fibroblastic, chondrogenic or adipogenic cell lineages; (3) fibro-adipogenic progenitors (FAPs) that can generate adipocytes and fibroblasts; (4) embryonic stem cells (ESCs); and (5) induced pluripotent stem cells (iPSCs) that can differentiate into any cell type [4].

Two different meat products are expected to be introduced to the market. Unstructured products such as burgers, sausages or nuggets will likely be commercialised first, and then structured products such as a chicken breast or a beefsteak will come later [5]. The manufactured tissue must resemble the in vivo tissue, reproducing morphological and functional characteristics, such as highly aligned muscle fibres and well-distributed fat [6].

2. Three-Dimensional Models for Cultivated Meat Production: Technological Aspects

Initially, in vitro studies maintained the cells in a two-dimensional (2D) culture, which allowed for an understanding of the biological mechanisms underlying cell functions, such as migration and differentiation. Over time, interdisciplinary improvements were made, and the application of biomaterials in culture was essential for the creation of a three-dimensional (3D) environment. It is possible to simulate variable and complex topographies in which cells can more closely mimic the behaviors of their in vivo environments [7][8].

Tissue construction aiming at successful recreation must mimic an extracellular matrix (ECM), including the composition, the physical properties and the technique for building a structure, all of which will affect the mechanical characteristics of the generated tissue [9]. It is also important to bear in mind that each tissue has a characteristic ECM, and different types of scaffolds can provide different cell targeting and differentiation outcomes [10]. Different scaffold architectures can be

achieved with existing techniques like porogen leaching, gas foaming, freeze-drying, electrospinning, 3D printing [11], sol-gel transition of gelatine [12] and 3D bioprinting (3DP) [13], among others.

CM is obtained through a process called cellular agriculture, which is based on tissue engineering principles [14]. Tissue engineering requires three technical components: cells, signals and scaffolds. In practice, it is first necessary to choose the best cell source and type. Second, a biocompatible tissue scaffold should be selected to provide structural support to those cells so they can proliferate and differentiate. Finally, but equally important, the necessary nutrients and small molecules must be provided; they will serve as external signals necessary for cell development.

2.1. Biomaterials

The biomaterials used in tissue engineering are commonly classified according to their origin as natural or synthetic. The natural biomaterials include chitosan, hyaluronic acid, fibrin, alginate, elastin, keratin, poly(hydroxybutyrate) (PHB) and decellularised extracellular matrix (dECM), among others. Some synthetic materials include polyglycolic acid (PGA), polylactic acid (PLA), poly DL-lactic co-glycolic acid (PLGA), polycaprolactone (PCL) and polyethylene glycol (PEG) [7][15].

The ideal material should be unlimited, biocompatible—to which cells can bind and interact with the extracellular proteins necessary to form the tissue—non-toxic and edible, which is one of the biggest challenges. Collagen is an animal-derived material used in a mixture with Matrigel and can be considered as a matrix or support. It enables differentiating myoblasts to align, compact and form a muscle fibre [16]. Plant-derived or synthetic polymers are an alternative to avoid using proteins from animal sources. The main difference between decellularised plant material and material of animal origin is the presence of ECM proteins. These proteins represent a mix of functional molecules such as collagen, fibronectin, glycosaminoglycans and a variety of growth factors that can influence cell development [17][18]. The absence of these molecules influences cell fixation and proliferation. Due to its biocompatibility, cellulose has arisen as a promising candidate for cell adhesion improvement [17]. However, other options are also viable: [19] showed successful myoblast cultivation in agarose, gellan and a xanthan–locust bean gum blend (XLB) as support materials with pea and soy protein additives.

As CM is designed for human consumption, compounds accepted by the FDA are favoured. The supply of these natural sources can guarantee availability for large-scale production. Among those already known are alginate, chitins and cellulose, which are widely used in food applications.

2.2. Microcarriers

Microcarriers have been used for quite some time in animal cell culture and they should be able to adapt to CM without major obstacles [20]. Although cell attachment is often a complex and empirical function, the interdependent factors are hydrophilicity, surface topography, net surface charge, charged group density, curvature and shear rates [21].

Cells attach and grow by apposition in microcarriers, which are beads ordinarily having a diameter of 100–200 µm [22]. Microcarriers differ in their physical properties such as size, porosity, rigidity, density and surface chemistry [23]. They can be made of synthetic or natural polymers. As mentioned, these microcarriers are applied to large-scale bioprocesses allowing for efficient proliferation of anchor-dependent cells. The advantages of this method are: easy production of large quantities of material and compatibility with various bioreactors and efficient proliferation of adherent cells. The disadvantages are the costs and potential inedibility [14]. For those that cannot become an integrated part of final product, the cells can be harvested from microcarriers by changing temperature, or through electronically induced shape change [22]. There is usually a significant cell/tissue yield lost independent of the dissociation process because the cell detachment is incomplete; this loss directly impacts the production efficiency and costs [23]. On a smaller scale, other alternatives to microcarrier cultures are being considered [24], such as spheroids [25][26], organoids [27] or single-cell suspension cultures [28].

2.3. Scaffolds

Scaffolds are 3D structures made to resemble the in vivo environment. These porous materials provide mechanical support and allow for an integrated network. Scaffolds are usually used to aid the differentiation step because they enable cells to adhere and mature into an edible meat product. Depending on the model type, scaffolds can grant potential vascularisation and spatial heterogeneity, essential features that the improve texture and structure of the final product, making it more like conventional meat [14].

Because CM is an edible product, the tissue scaffolds should be biodegradable and non-toxic. However, in some cases, they may be designed to be degraded or removed before consumption [4]. The scaffolds may also have appropriate

mechanical properties, including strength, thickness, stiffness, pore size, texture and architecture [14]. For example, porosity directly influences media perfusion, and tissue maturation for a similarity with conventional meat scaffolds also need to support tissue maturation beyond a thickness of 1 cm [29]. Other properties such as nutritional value, thermal stability, non-allergenicity, non-toxicity and the ability to improve organoleptic properties are important items to consider when choosing the best scaffold depending on the final desired product [14].

Currently, there is no commercially available scaffold for CM that is free of animal biomaterials, although a scaffold could be derived from natural, synthetic or composite biomaterials. Natural materials can be derived from animal sources, such as collagen, fibrin and hyaluronic acid, or from plant origin, like alginate, decellularised plant materials and cellulose. Other sources of scaffold materials include chitosan from crustaceans, yeast or fungi and fungal mycelium. Synthetic polymers include a range of polyesters (polyamide and polyethylene). Scaffolds made of these materials can be food safe. Synthetic polymers such as PLA and PLGA are degraded by chemical hydrolysis to generate products like lactic acid and glycolic acid, which are considered safe in food [29].

Textured soy protein (TSP) is an important potential scaffold to consider. It is a porous, food-grade, inexpensive by-product of soybean oil processing that has great nutritional value and high protein content in addition to improving the final texture of the product. Due to its characteristics, it is commonly used in plant-based meat substitutes and does not require major modifications; hence, it is highly applicable for mass production. TSP can be adapted to various sizes and shapes, thus facilitating implementation in cultivation processes—for example, in bioreactors [30].

Other materials have characteristics that have attracted attention for scaffold production. A group in Korea fabricated microspheres of gelatine (GMS) to use as a scaffold with a high surface-area-to-volume ratio for CM. Gelatine is an edible material capable of promoting cell adhesion through its tripeptide Arg-Gly-Asp (RGD) sequence. In addition to being a collagen-derived natural polymer, its other advantages include high biocompatibility, biodegradability and processability [31]. Meat analogues with a suitable scaffold made of gelatine fibres can be produced through dry-jet wet-spinning, producing 3D aligned tissues. Aortic smooth muscle cells and skeletal muscle myoblasts have been cultivated in gelatine fibre scaffolds, which are safe and edible materials [32].

3. Assembly Methods

Functional tissues require characteristics such as mechanical stiffness and chemical and surface properties for desirable cellular interactions to trigger cell responses. Because scaffolds are important for mimicking the complex spatiotemporal distribution of in vivo tissue, some assembly methods used in tissue engineering to manufacture such structures have been developed [6].

3.1. Cell Layering or Self-Assembly

Layer-by-layer (LbL) assembly is a highly adjustable and simple multilayer self-assembly technique. It is possible to produce multilayer coatings with a specific architecture and composition from an extensive catalogue of available materials for several biomedical applications [33]. This production process is fast and scalable, and it can manufacture highly dense, multicellular and textured tissues in normal culture plates without a bioreactor. A bioreactor is necessary if the final product needs to be thicker [32].

There are three basic methods for cell layering: stacking cell sheets, rolling a cohesive tissue sheet and in situ deposition of cell-laden biomaterials. The first one uses a temperature-responsive polymer-coated culture dish to form a multi-layered tissue. The second method consists of wrapping a whole piece of a thin tissue sheet around a tubular support and culturing until tissue fusion. For the third approach, a handheld apparatus is used to deposit cell-laden biomaterials [6].

Biomaterials are used to promote or prevent cell adhesion, to maintain or direct cellular phenotypes, and to provide 3D structures for cell culture or co-culture. The range of biomaterials applied for LbL assembly include biomolecules, polyelectrolytes, particles and colloids, among others [33]. The successful co-culture of myoblasts and preadipocytes has already demonstrated the feasibility of this approach for building meat-like tissues of any size and thickness. Scaffolds do not need to be used because the cells produce their own ECM that is preserved and makes robust sheets [32].

3.2. Spinning

Spinning refers to a manufacturing process for creating fibre-shaped materials and can be applied in various manufacturing fields, including tissue engineering [34]. This material is a potential choice for in vitro tissue production because it produces highly aligned structures with long lengths and flexibility, features that produce functional and

morphological characteristics. Among the spinning methods, wet spinning and electrospinning are suitable for this [6]. Wet spinning is commonly used to produce fibres with micron diameters by using polymers dissolved in non-volatile or heat-unstable solvents. Electrospinning enables the production of nanofibers that can meet the functional requirements of tolerating high temperatures and demonstrating strong absorption for filtration [35].

Wet-spun fibres allow for cell adhesion and proliferation into highly oriented porous structures. For this approach, diverse biomaterials can be used as polymers, such as PLGA, chitosan and alginate [34]. Cells can be mixed with biopolymers and laden or encapsulated within the polymer through microfluidics devices, forming cell-laden fibres. It is possible to assemble larger scaffolds or tissues with this methodology. The main advantage of this method for CM production is the ability to modulate the thickness, shape and mechanical rigidity of the fibres through microfluidic channels [6].

Electrospinning is based on the use of electrical forces to produce fibres, which provide a large surface-area-to-volume ratio, thus improving cellular development. Biopolymers for cell electrospinning can also be natural or synthetic polymers [36]. Electrospun scaffolds are also very interesting for CM production because this technique generates alignment cues that guide the uniaxial alignment of seeded cells. Ref. [37] demonstrated that even after electrospinning, myoblasts on fibrin scaffolds exhibited a uniform distribution, and they continued to proliferate and differentiate.

3.3. Bioprinting

3DP is a promising tissue engineering technique for simulating the structural characteristics of meat. The advantages of 3D bioprinting are the ability to control the structure and composition of a product in addition to its potential scalability (Kang et al. 2021). 3DP is an advanced additive manufacturing platform that allows for the pre-defined deposition of cells, biomaterials and growth factors. It is based on computer-aided design and manufacturing (CAD/CAM), which customises the layer-by-layer printing process with a high level of flexibility and reproducibility. 3DP is an emerging technology that has attracted increased attention in the last few years in the food field due to its applicability for sustainably manufacturing customised products with intricate shapes and textures [38]. It also allows for improving the nutritional profile and sensorial values of the product [39].

The 3DP production process allows for the deposition of materials or inks in a layer-by-layer fashion to generate complex 3D structures that resemble laboratory cultured cells [39]. The most-used 3DP methods are extrusion, inkjet printing, binder jetting and bioprinting. For meat product fabrication, the extrusion method is commonly used to print the 3D structures [38].

Bio-inks consist of cells, biomaterials and other molecules such as growth factors. The medium for the cell suspension contains polymer crosslinkers, such as $CaCl_2$, thrombin, salt (NaCl), gelatine and fibrinogen. Biomaterials, such as melt-cure polymers, hydrogels or dECM, are utilised as scaffolds in bio-inks to provide an appropriate microenvironment for cell adhesion, migration and differentiation [1][6][40]. Therefore, 3DP provides the possibility for reshaping the structure of conventional meat: the structure can be designed in such a way that raw materials can be thoroughly mixed and organised. With this process, it is possible to fabricate flexible artificial vessels and to control the graininess and toughness of the final product, to ensure that it is similar to conventional meat [20].

Printed constructs enable nutrient diffusion and enhance porosity. Researchers have described successful printing of engineered tissues, including skeletal muscle [41][42][43]. Obviously, 3D-printed CM has benefited from advances made in the tissue engineering field. Although it does not need a complex vascular system like in natural tissue, more research is required to improve printable, non-animal materials and potentially edible scaffold compositions.

Ianovici et al. (2022) [1] tested two 3D-printed scaffolding compositions, not derived from animal biomaterials and enriched with plant proteins, for bovine satellite cell cultivation. They evaluated mixtures of pea protein isolate (PPI) and soy protein isolate (SPI) with RGD-modified alginate suitable for flexible 3DP and cell cultivation. They observed bovine satellite cell attachment, spreading, maturation and differentiation. They applied extrusion using an edible, removable agar support bath. PPI-enriched bio-inks allowed for cellular bioprinting.

Kang et al. (2021) [44] produced the first whole-cut CM-like tissue. It was composed of three types of primary bovine cells (satellite cells, adipose-derived stem cells and endothelial cells). The authors used tendon-gel-integrated bioprinting (TIP) to fabricate cell fibres. They then modelled the subsequent cell differentiation into the structure of real meat. When assembled, it mimicked the histological structures of a real steak. Despite its good appearance, the meat-like tissue was very small and not edible, indicating that more research is needed for improvement. Although 3DP is likely to achieve a

final product with a thickness close to that of real meat, it might be less amenable to the scaling required to achieve CM production.

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