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Cultured Meat Scaffolding: Contemporary Synergistic Methods

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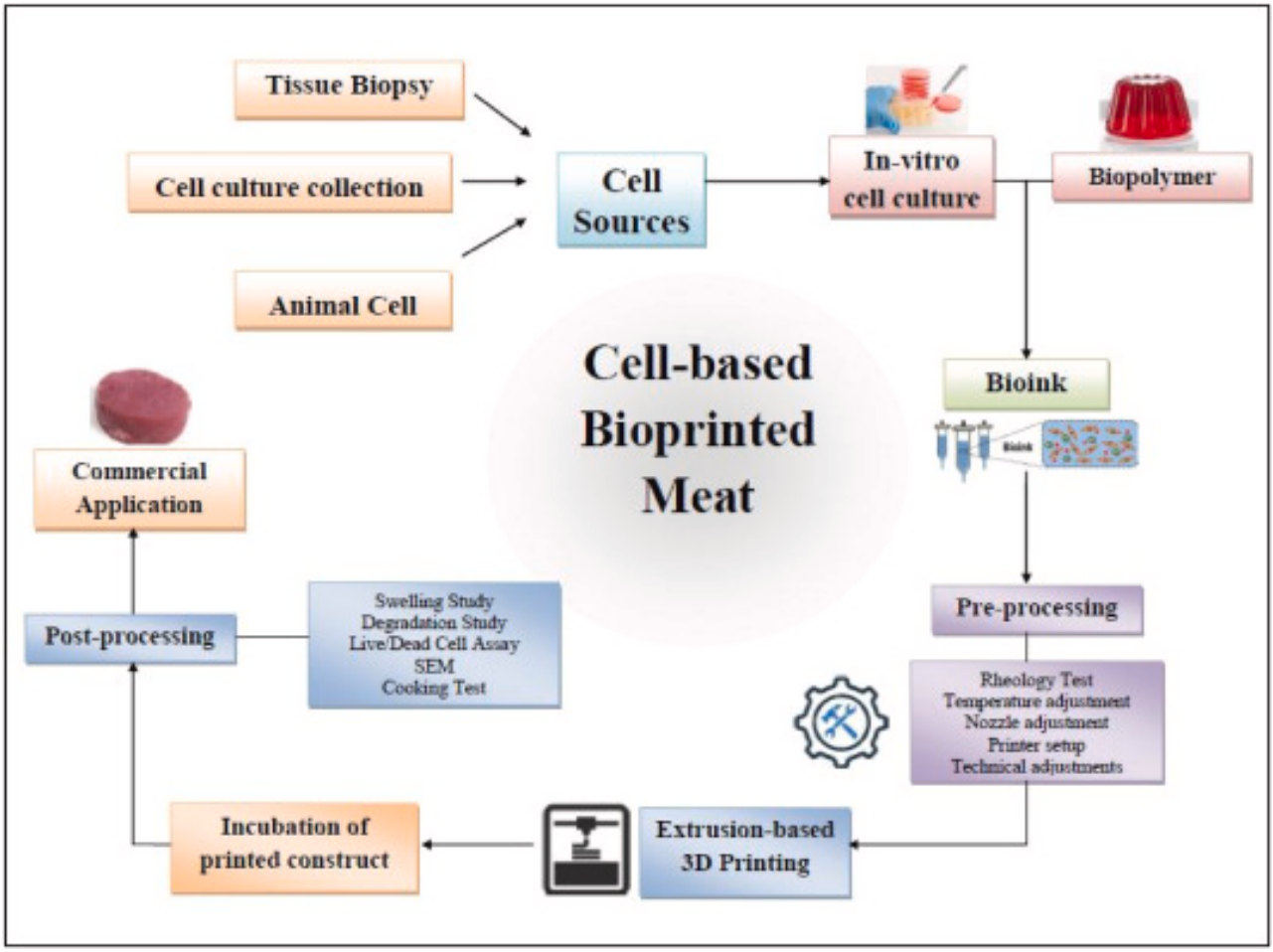

Adapted from Tibrewal et al. (2023).

Abstract

Traditional meat is a reliably global source of protein fulfilling dietary needs, but produces great negative externalities which may not be sustainable in the long-term. Cultured meat aims to reproduce traditional meat in such a way that it is competitively attractive for consumers, economically viable and environmentally advantageous. Cultivated meat is theorized to be able to produce 1 billion beef burgers from a single cow biopsy, which could mean a great decrease of animals used in the industry and subsequent environmental damages. For the viability of cultured meat, multiple aspects need to be addressed, however. One key aspect for the creation of properly structured meat is the use of scaffolds, which give the cells of cultured meat an extracellular matrix-like structure to adhere to, differentiate on and proliferate on. Here we report the multitudinous nature of the cultured meat industry, specifically the scaffolding aspect. We analyze contemporary methods used to scaffold cultured meat. Finally we propose the synergistic implementation of microcarriers, hydrogels and 3D printing as a viable current-day implementation for the production of cultured meat.

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Foreword

In this paper we aim to not only consider the science behind cultured meat, but take into account important aspects such as environmentalism and economic viability. Such a diverse approach is difficult, so here we report mainly the scientific results, which we have screened for economic and environmental viability.

1.0. Introduction

World population is on an ever high level and is continuing to rise while the herd size of domesticated farm animals can increase by tens of percentages per capita by the year 2050 (*FAO*, 2018). This livestock provides 18% of calories for humans, while taking up 77% of global farmland (*Cultivated Meat | Resource Guide (2021) | The Good Food Institute*, 2021; Ritchie & Roser, 2013). While more sustainable food sources than meat derived from livestock are available, often the high quality of protein is hard to replace. Research has shown that cultured meat (CM) can offer a sustainable way to produce food which is high in quality protein, being theorized to be able to produce 1 billion beef burgers from a single cow biopsy (Kumar et al., 2021), offering a solution to these problems (Shaikh et al., 2021).

CM, also called lab-grown meat or cultivated meat, offers the option to produce meat without this large-scale use of farmland by domestic animals. CM falls within the field of cellular agriculture, which aims to cellularly produce materials such as leather, enzymes and tissue. CM is a technological field of which the terminology and meaning of the field is still changing (Stephens et al., 2018), the variability of the terms used to describe the process reflecting the ongoing development of the field. The term ‘cultured meat’ is shown to be most scientifically correct and appealing (Bomkamp et al., 2022). This developing field might reshape supply chains and livelihoods on a global scale and has a far greater potential for efficient production of meat, using land an estimated 2000 to 4000 percent more efficiently in the case of beef production (*The Science of Cultivated Meat | GFI*, 2021).

Recent review articles shed light on the multi-faceted differences between CM and traditional meat. Most notably, the drastically different production method of CM offers certain advantages over the animal farming of traditional meat, such as lower greenhouse gas emissions, higher manipulability of the composition of the meat, faster production and lower land requirements. Next to these quantifiable advantages, there are also more subjective advantages, such as the avoidance of animals harmed during the production of traditional meat.However, the industry of CM faces multiple challenges. The most notable of these challenges are the high production costs, reluctant consumer acceptability and high energy costs (Hong et al., 2021). It is clear that this CM offers a potential solution to the polluting nature of traditional meat, but the technology is far from perfect and is accompanied by many challenges.

One of the largest challenges of CM is consumer acceptability, which links to the production process. The general process of CM production involves the steps of cell extraction, cell line

establishment, cell culturing and scaffolding. Cells with the potency to develop into meat must be extracted, after which they are isolated and cultured within the proper growth medium. Within this paper, we focus on the techniques used for CM to resemble traditional meat, namely the scaffolding step. CM aspires to mimic traditional meat, since the mimicking of the aspects of traditional meat is an important factor for the consumer acceptance and ultimate success of the CM industry (Stephens et al., 2018). Scaffolding within the field of CM includes a wide range of techniques to allow cells to adhere to a structure resembling the extracellular matrix (ECM), which can allow growth, differentiation and migration. Ultimately, these scaffolds facilitate the growth of the cells used to produce CM and aims to let these cells proliferate in such a way that they constitute a structure resembling traditional meat.

Here we describe the general mechanisms of CM production with an emphasis on the scaffolding step. This scaffolding step is investigated by researching the properties of scaffolds and looking at the used methods within the biotechnological industry. After this background is established, we elaborate on the mechanotransductive role of scaffolds, which plays a role in the prevention of anoikis and the structuring of CM. From recent literature we analyze popular scaffold techniques and argue the possible combination techniques. Ultimately, we discuss the viability of these aspects, specifically the combination of microcarriers, hydrogels and 3D bioprinting to grow and structure CM effectively.

2.0 The cells of CM

Though many of the methods of CM production share a similar sequence of steps, the exact mechanisms and techniques used vary. These different biological techniques can include the use of self-assembling organoids, iPSCs or direct use of stem cells, for example. The general process of CM production involves the selection of cells, growth in bioreactors, growth medium for timed differentiation and scaffolding for structure of the meat (Figure 1)(*The Science of Cultivated Meat* | *GFI*, 2021).

Each of these steps include their challenges and possibilities. Financially, the biggest challenges of these steps are indicated to be the growth medium, bioreactors and the labor associated with production, accounting for 80% of the cost (Garrison et al., 2022). Another key challenge is the recreation of a product which achieves high consumer acceptance, for which the scaffolding step is most important. To delve into the different mechanisms of production, this paper will start investigating the different cell types used to instigate this process of CM production.

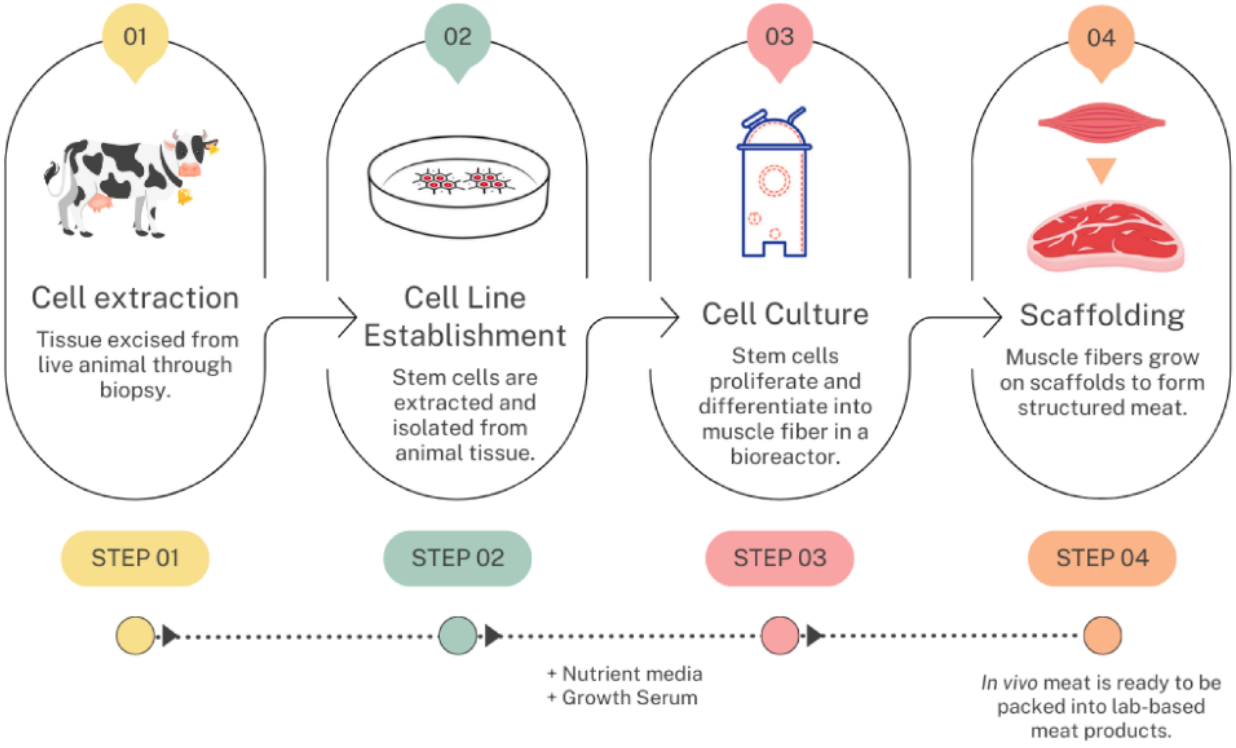

Figure 1, adapted from Ching et al. (2022). The general process of CM production involves the steps of cell extraction, followed by the establishment of cell lines, cell culturing and ultimately the use of scaffolds to structure the cell to constitute meat that resembles traditional meat. Details of these steps influence the product. Different cell types, growth media and types of scaffolds can be used, impacting the final product.

2.1 Traditional meat cell types

While we do not discuss the intricate structure of muscle, it is important to discuss that there are a variety of cells which compose meat. The most important cells within traditional meat are myocytes, which form myofibers. Other cell types important are adipocytes and fibroblasts, which can be used during the production of CM to enhance the grown meat. As will be discussed extensively in further sections, the ECM is a critical component for the development of myocytes to form muscle (Chapman et al., 2016), fibroblasts facilitate this. Importantly, CM tries to mimic skeletal muscle. There is a variety of adipocyte tissue in and around traditional meat (Vettor et al., 2009). This could play an important factor in the mimicking of traditional meat by CM.

2.2 Cells types within CM production

The types and amount of cell lines used by CM manufacturing companies vary greatly. A 2020 survey questionnaire for companies of this specific industry has shown this complexity (figure 2). While many different cell types can be used, currently myosatellite/myoblast (MySCs), fibroblast or mesenchymal stem/stromal (MSCs) cells were most popularly used (Swartz et al., 2020).

Embryonic stem cells (ESCs) are promising due their pluripotency, being derived from the inner cell mass of blastocysts. ESC cell lines for CM are not properly established however, as such ESCs derived from animal species such as bovine species have only been established in 2018. Additionally, such cell lines are sensitive to growth factors and the growth factors during their differentiation into myocytes, for example, must be carefully established. Induced pluripotent stem cells (iPSCs) are more commonly used since their utility is generally the same as ESCs, but the cell lines are easier to derive than ESCs (Choi et al., 2015).

MSCs, while having a lesser degree of pluripotency than iPSCs, are commonly used for the production of CM, due to their ability to self-renew and differentiate (Shaikh et al., 2021). MSCs form a supply of stem cells to replenish cell populations, such as osteoblasts, adipocytes and chondrocytes. MSCs also can have the ability to differentiate into myocytes. MSCs however have a larger variance of cell phenotypes, which depends on the tissue they are derived from, meaning that MSCs must be selected which can differentiate into the myocytes necessary for CM.

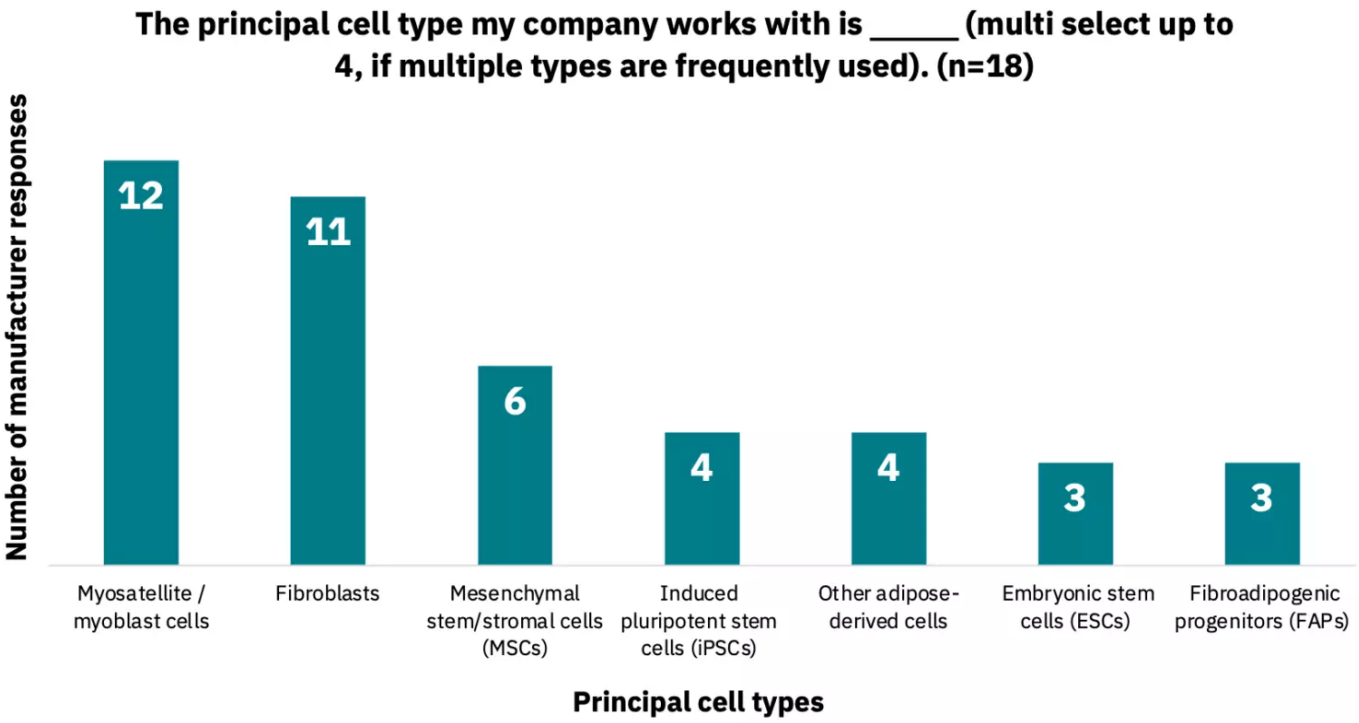

Figure 2, Adapted from Swartz et al. (2020). The most prominently used cell types for CM production, as concluded from the 2020 cultivated meat survey (Bomkamp, et al., 2022). Most popularly used are the MySCs, fibroblasts and MSCs. Combinations of these cells could be used to optimally produce CM, such as through the co-culturing of fibroblasts and MySCs (Kino-oka et al., 2013).

Fibroblasts and fibroadipogenic progenitors, a type of mesenchymal stem cell, are used because of their ability to contribute to the ECM. Fibroblasts can be co-cultured together with MySCs, which has been argumented to be an efficient way to develop CM. During such a co-culture, the emphasis must be put on the growth of the MySCs, by inhibiting fibroblast growth (Kino-oka et al., 2013).

MySCs are most popularly used to produce CM. MySCs are cells which are abundantly found along the myofibers under the basal lamina of muscle. They can be derived from muscle tissue after a small biopsy or by harvesting the cells of a deceased animal (Liu et al., 2015). Since these cells originate from muscle, they can be used directly for the development of myoblasts (Pajalunga & Crescenzi, 2021) and ultimately muscle tissue. However, maintaining the pluripotency of these stem cells in vitro is a challenge. Furthermore, while effective to produce cells originating from myoblasts, other cell types within muscle might not be possible to be developed from this stem cell.

2.3 Cell growth medium

Cost analysis of CM shows that reduced costs of growth medium can greatly influence the price of CM (Garrison et al., 2022), making growth medium an important consideration. Such a growth medium requires a wide range of compounds, such as amino acids, salts and carbon energy sources (*Cultivated Meat Cell Culture Media | Deep Dive | GFI*, 2021). In this paper we forego details of this step in favor of the scaffolding step. We refer to (*Cultivated Meat Cell Culture Media | Deep Dive | GFI*, 2021) for a detailed overview of the cell growth media used for CM.

3.0 The properties of scaffolding

CM producers aspire CM to resemble natural meat, since the unnatural aspect of CM makes this a technology and collection of products more difficult to accept for consumers (Stephens et al., 2018). Technologies have developed to stimulate the aforementioned cells to be structured in a similar fashion. In order for CM to resemble natural meat, scaffolds are used to structure the CM cells, such scaffolds are used to give the different cell types used within the CM production a platform to attach to, permitting differentiation and maturation and establishing a structure (Seah et al., 2022). In this section, we dive into the general background of scaffolding used within the production of CM. In later sections we elaborate on these aspects by looking at the molecular mechanisms of the techniques used to solve problems regarding the production of CM.

3.1 General properties

There are different qualities to be considered for the scaffolds used for CM production. A first logical consideration would be if the scaffold will stay in the CM or will be removed. Tissue engineering (TE) is a field which has developed the use of scaffolding for a longer amount of time, which is why this field offers good insights into the qualities appropriate for scaffolds used within CM production. Scaffolding within the field of CM is thus inspired by the TE field. Since CM is eaten and many tissues within the field of TE most often are not, certain qualities need to be adjusted to make the tissue of CM fitting for consumption. According to Seah et al (2022), there are four key aspects that need to be taken into account for tissue engineering (TE), of which the mechanisms are similar to scaffolds being used for CM production. These 4 aspects are the biocompatibility, biodegradability, architecture of the scaffold and the technology used to manufacture the scaffold (table 1).

Table 1, different properties of CM scaffolds and their details.

Property	Details
Biocompatibility	The scaffold must in first instance be compatible with the cells used. The scaffold must facilitate tissue formation, which includes adherence and possibly migration. Also, the cells must not have a detrimental (immune) reaction, which could interfere with tissue formation. Specifically for CM, this is a limitation within the domain of scaffolding, since the requirements for edibility limit the options in terms of materials used (Reiss et al., 2021).
Biodegradability	If a scaffold is used which is not mechanically removed from the tissue, it should either be degradable or in the case of CM edible.
Scaffold architecture	The scaffold used to grow the CM should mimic the vascularization of blood vessels, allow the supply of nutrients and the clearance of waste materials. The scaffold should thus be porous, allowing for the diffusive exchange of materials from within and outside of the construct. Furthermore, the pore and scaffold size need to be taken into account. Collagen could be used as a material for the scaffold creation, since this is a natural product which also naturally possesses the ligand necessary for cell binding. If the scaffolding is made synthetically, the synthetical material should include binding regions for the cells.
Manufacturing technology	Preferably this scaffold is as cost-efficient as possible. Generally, the production of the scaffolds individually or in batches can influence the price, next to the materials used. For this paper we focus on the non-economical aspects of the scaffolding.

3.2 Scaffolding methodologies

There is a wide variety of techniques used for scaffolding within the field of CM research (Figure 3). Often, different techniques can be used, sometimes in tandem. The different techniques include the use of microcarriers, porous scaffolds, hydrogels, 3D printing and fiber scaffolds. These techniques can be categorized within top-down and bottom-up approaches. The top-down approach aims to produce a scaffold, onto which cells are seeded and grown, such as hydrogels. A bottom-up approach aims to create a more complex structure, e.g. through the use of 3D printing. Within these categories and within these different methods for scaffolding, there are many different possibilities. This wide range of techniques is accompanied by an equally diverse range of challenges (Levi et al., 2022). The range of techniques can be further categorized regarding scaffold material and the type (Bomkamp et al., 2022), which includes the use of synthetic polymers, ECM and animal-based proteins, plant or fungus-based materials and self-assembling peptides.

This wide range of combinations within the CM biotechnological field shows its complexity and all the possibilities within this field. Within the scaffold material categories, a wide range of materials is available. It would be counterproductive if the materials would rely on animals, since this is exactly what the technology of CM could replace. Hence, the focus should be to find the materials which do not rely on animals and also allow low-cost production of CM while maintaining the viability of CM production.

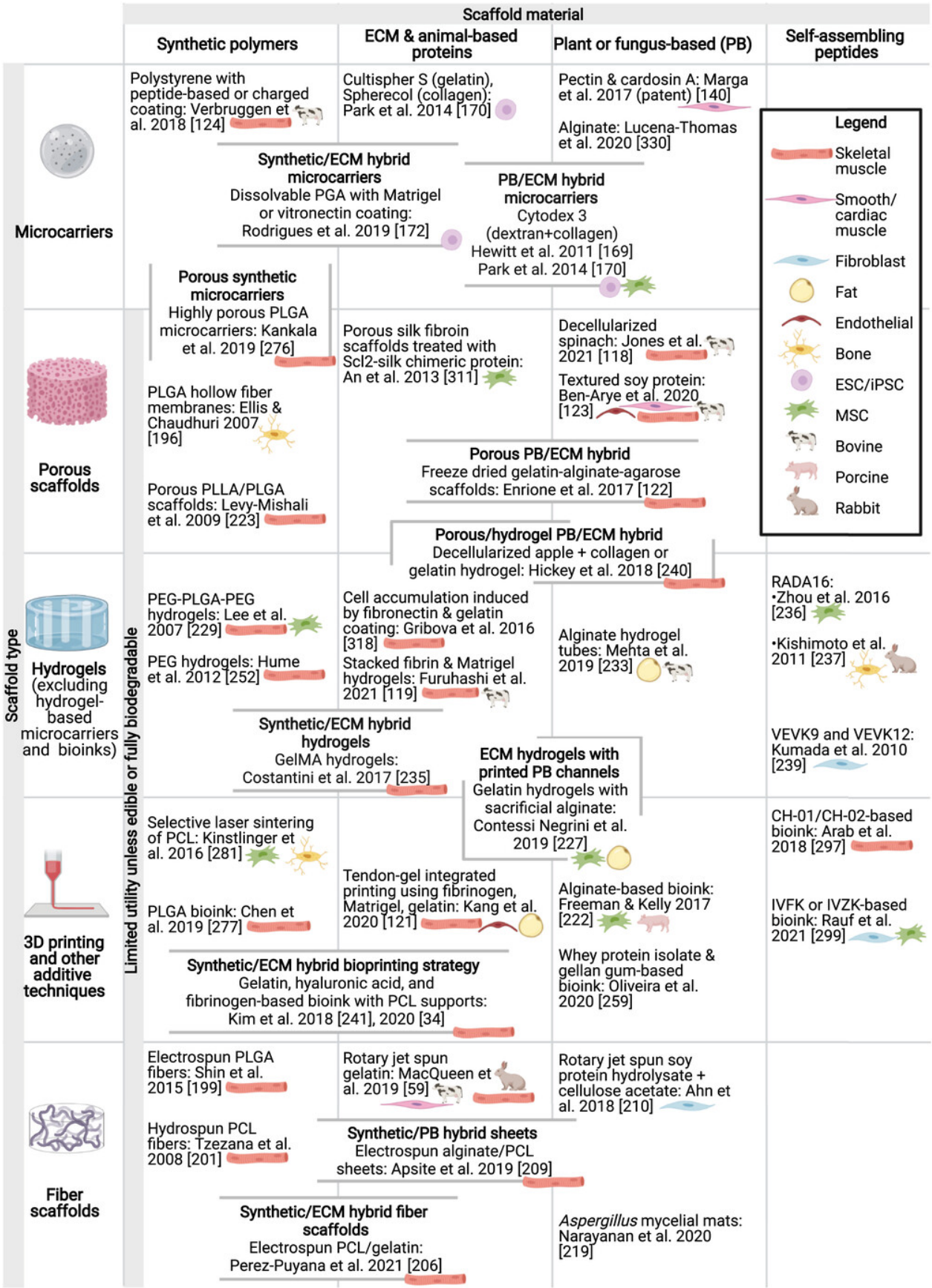
Scaffold material
Synthetic polymers
ECM & animal-based proteins
Plant or fungus-based (PB)
Self-assembling peptides
Scaffold type
Limited utility unless edible or fully biodegradable
Microcarriers
Porous scaffolds
Hydrogels (excluding hydrogel-based microcarriers and bioinks)
3D printing and other additive techniques
Fiber scaffolds
Polystyrene with peptide-based or charged coating: Verbruggen et al. 2018 [124]
Cultispher S (gelatin), Spherecol (collagen): Park et al. 2014 [170]
Pectin & cardosin A: Marga et al. 2017 (patent) [140]
Alginate: Lucena-Thomas et al. 2020 [330]
Synthetic/ECM hybrid microcarriers
Dissolvable PGA with Matrigel or vitronectin coating: Rodrigues et al. 2019 [172]
PB/ECM hybrid microcarriers
Cytodex 3 (dextran+collagen)
Hewitt et al. 2011 [169]
Park et al. 2014 [170]
Porous synthetic microcarriers
Highly porous PLGA microcarriers: Kankala et al. 2019 [276]
Porous silk fibroin scaffolds treated with Scl2-silk chimeric protein: An et al. 2013 [311]
Decellularized spinach: Jones et al. 2021 [118]
Textured soy protein: Ben-Arye et al. 2020 [123]
PLGA hollow fiber membranes: Ellis & Chaudhuri 2007 [196]
Porous PB/ECM hybrid
Freeze dried gelatin-alginate-agarose scaffolds: Enrione et al. 2017 [122]
Porous PLLA/PLGA scaffolds: Levy-Mishali et al. 2009 [223]
Porous/hydrogel PB/ECM hybrid
Decellularized apple + collagen or gelatin hydrogel: Hickey et al. 2018 [240]
PEG-PLGA-PEG hydrogels: Lee et al. 2007 [229]
Cell accumulation induced by fibronectin & gelatin coating: Gribova et al. 2016 [318]
Alginate hydrogel tubes: Mehta et al. 2019 [233]
RADA16:
•Zhou et al. 2016 [236]
•Kishimoto et al. 2011 [237]
PEG hydrogels: Hume et al. 2012 [252]
Stacked fibrin & Matrigel hydrogels: Furuhashi et al. 2021 [119]
Synthetic/ECM hybrid hydrogels
GelMA hydrogels: Costantini et al. 2017 [235]
ECM hydrogels with printed PB channels
Gelatin hydrogels with sacrificial alginate: Contessi Negrini et al. 2019 [227]
VEVK9 and VEVK12: Kumada et al. 2010 [239]
Selective laser sintering of PCL: Kinstlinger et al. 2016 [281]
CH-01/CH-02-based bioink: Arab et al. 2018 [297]
Tendon-gel integrated printing using fibrinogen, Matrigel, gelatin: Kang et al. 2020 [121]
Alginate-based bioink: Freeman & Kelly 2017 [222]
PLGA bioink: Chen et al. 2019 [277]
IVFK or IVZK-based bioink: Rauf et al. 2021 [299]
Synthetic/ECM hybrid bioprinting strategy
Gelatin, hyaluronic acid, and fibrinogen-based bioink with PCL supports: Kim et al. 2018 [241], 2020 [34]
Whey protein isolate & gellan gum-based bioink: Oliveira et al. 2020 [259]
Electrospun PLGA fibers: Shin et al. 2015 [199]
Rotary jet spun gelatin: MacQueen et al. 2019 [59]
Rotary jet spun soy protein hydrolysate + cellulose acetate: Ahn et al. 2018 [210]
Hydrospun PCL fibers: Tzezana et al. 2008 [201]
Synthetic/PB hybrid sheets
Electrospun alginate/PCL sheets: Apsite et al. 2019 [209]
Synthetic/ECM hybrid fiber scaffolds
Electrospun PCL/gelatin: Perez-Puyana et al. 2021 [206]
Aspergillus mycelial mats: Narayanan et al. 2020 [219]
Legend
Skeletal muscle
Smooth/ cardiac muscle
Fibroblast
Fat
Endothelial
Bone
ESC/iPSC
MSC
Bovine
Porcine
Rabbit

Figure 3, adapted from Bomkamp et al. (2022). This illustration summarizes the different possibilities within the CM biotechnological field in regard to scaffolding. Different techniques and combinations thereof are categorized according to their scaffold type(s) and material(s). Within multiple cases a combination of different scaffold types and/or materials is used, as shown by the techniques with an adjusted gray outline. The symbols on the bottom right of each method shows the types of cells used within the testing of the respectives method. This figure is one way of illustrating the different scaffolding methods. While this figure does not depict all of the most recent developments within the field, it illustrates effectively the different options within CM scaffolding. For a more detailed overview, we refer to Bomkamp et al. (2022).

4.0 Scaffolding, an artificial ECM

In normal physiological environments, muscle cells develop surrounded by the stiffness of the ECM. The ECM is necessary for these cells, leading to downstream effects of this signaling include differentiation, polarization, migration and tissue homeostasis (*Cultivated Meat Scaffolding* | *Deep Dive* | *GFI*, 2021a; Handorf et al., 2015; Sun et al., 2016). Ultimately this signaling is caused by the allosteric effect the forces of the ECM have on the cells. This signaling between the ECM and cells is facilitated by connective points called focal adhesion complexes, connecting the actomyosin cytoskeleton to the surrounding ECM. The composition of the ECM is different per type of tissue, and should mimic the environment of muscle cells to let muscle cells develop naturally. The main composing element of ECM is collagen, which gives the matrix its mechanical strength. Additionally, proteoglycan can sequester growth factors while glycoprotein can connect muscle cells to the basement membrane (Gillies & Lieber, 2011). The composition of the ECM relates to the chemical and the mechanical effects the ECM has on cells, which depicts a different niche per tissue type (Gattazzo et al., 2014).

4.1 Mechanotransduction and CM

Specific cell membrane proteins called integrins connect the ECM and the structure of the cell's cytoskeleton, allowing for mechanotransduction and subsequent signaling (*Cultivated Meat Scaffolding* | *Deep Dive* | *GFI*, 2021). These integrins are important cell adhesion molecules, and are accompanied by adaptor proteins (Paolo Boffetta & Hainaut Pierre, 2019). The physical force applied by the ECM on the cells is exerted on these integrins, and since the integrins, cytoskeleton and also the nuclear matrix are connected, the force is bi-directionaly transduced through these cellular components (Harburger & Calderwood, 2009). The significance of this is that chromatin in the nuclear matrix is affected, causing changes in gene expression. The change of this gene expression, which sometimes relates to the development of these cells, then causes these cells to be able to change the surrounding ECM (Bissell et al., 1982). This process can be called "dynamic reciprocity", which could be an important consideration when designing scaffolds to properly develop CM.

The scaffolding used for the production of CM substitutes functions of the ECM, which offers both structure and mechanical signals to cells. Importantly for the production of CM, the scaffold, mimicking the ECM, can specifically play a role in the regulation of pluripotency and differentiation of cells. As discussed before the use of stem cells is common practise when producing CM, which are especially sensitive to signals which regulate pluripotency and differentiation. ECM, which has an elastic property, directs stem cells towards differentiation. Neurogenesis can be a result for example, while a stiffer matrix can lead cells towards myogenesis (Engler et al., 2006). All in all, the ECM plays, both chemically and mechanically, a large role in myogenesis. The scaffolding used to produce CM should thus mimic these in vivo conditions. Another important aspect of the ECM is to prevent ‘anoikis’, which is a term used to describe apoptosis induced by a lack of ECM signaling. (Frisch & Screaton, 2001).

Mimicking the ECM through the use of scaffolding is necessary to signal cells used to produce CM to differentiate into muscle. Anchorage-dependent cells rely on the integrin-signaling pathway for survival and proliferation (Schwartz, 1997). Many of the cell types used for the production of CM such as myocytes (Figure 4), are anchorage-dependent and thus rely on this signaling pathway (Reiss et al., 2021). It is important to note that this anchorage is not limited to the ECM, since cell-cell interactions can provide similar signals, for example through cadherin binding (Paolo Boffetta & Hainaut Pierre, 2019). Integrin connects the ECM to the cytoskeleton at regions called focal adhesion complexes, which are key for the integrin-signaling pathway. These proteins are heterodimers of non-covalently associated α and subunits, which both are type I single-pass transmembrane proteins. The extracellular domains of the complex can bind to ligands of the ECM or counter-receptors on other cells (Harburger & Calderwood, 2009). Intracellular domains consist of a generally short tail which can bind and associate with various intracellular proteins, thereby facilitating the bi-directional transfer of force across the cell membrane. Integrins bound to the ECM cause the recruitment and binding of various scaffolding proteins, which connect the integrins to the actin cytoskeleton (Figure 4) (Giannone & Sheetz, 2006). Through these factors integrins can give signals which the cell can use together with signals from other sources, such as those from G-protein-coupled receptors. Proper mechanical interaction between the ECM and cells due to mechanotransduction through integrins causes factors, which can instigate apoptosis, to be sequestered. Such factors include the protein BH-3, of which the domain Bmf binds to the cytoskeleton (Frisch & Screaton, 2001).

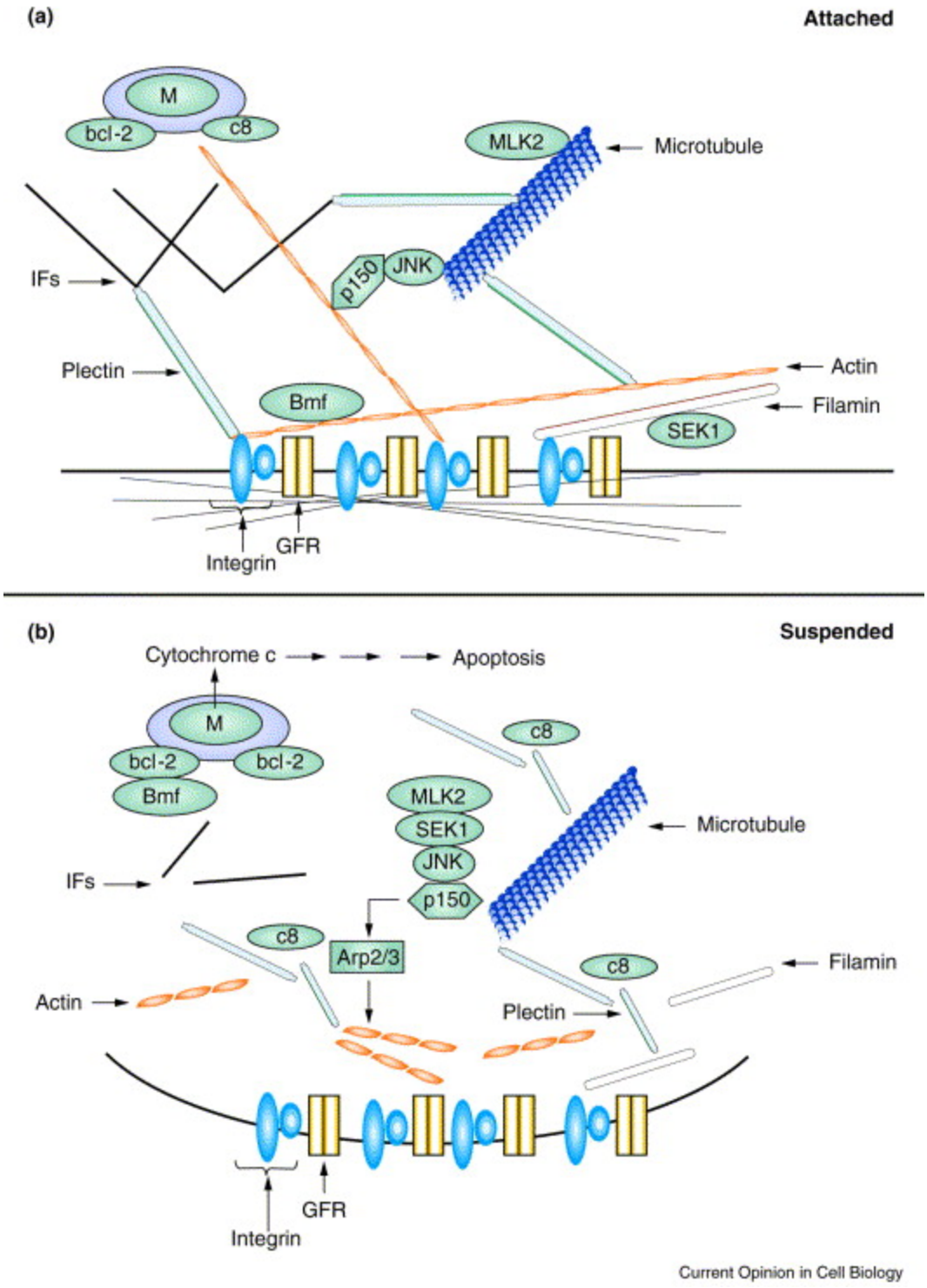

Figure 4, adapted from Frisch & Screaton (2001). A) Under normal physiological conditions where cells are properly able to associate to ligands of the ECM, many factors bind to the cytoskeleton. In this case the microtubules, intermediate filaments (IFs) and actin are bound together. Due to this binding, BH-3, canonically a mitochondrial apoptosis related protein, can bind through its Bmf domain to the cytoskeleton. JNK and SEK1, part of the JNK pathway, and

MLK2 are sequestered similarly due to the binding possible to the cytoskeleton. Furthermore, TNFR2 may also be kept bound to the cytoskeleton through the binding to intermediate filaments and p150-Spir can stabilize actin filaments. B) Suspended cells unbound to an ECM anoikis may be induced due to the release of the previously mentioned proteins. The theorized mechanism is that the release of the domain Bmf, is able to bind to bcl-2. Caspase-8, normally bound to the mitochondria, can now be released together with cytochrome c and apoptosis is induced (Frisch & Screaton, 2001).

Absence of the attachment of integrin to substrates of the ECM can lead to the induction of anoikis, which takes places through apoptotic pathways. The absence of connection between integrin and its extracellular ligands can lead to changes in cytoskeletal dynamics, which can lead to the permeabilization of the outer mitochondrial membrane. Apoptosis may also be induced through the extrinsic apoptotic pathway including the upregulation of factors for the TNFR2. In both cases, caspases can be activated and ultimately induce cell death (Paolo Boffetta & Hainaut Pierre, 2019).

5.0 Microcarriers

Since the (stem) cells used for the production of CM need to adhere to an ECM-like surface, this needs to be recreated for a higher efficiency of growth. Microcarriers are small beads ranging in size from 100-300µm which can solve multiple problems related to the production of CM (McKee & Chaudhry, 2017). Microcarriers are structures resembling beads, containing the appropriate ligands for cell types used within a biotechnological process to grow on. These beads can thus be used during this process to increase growth efficiency, while allowing differentiation (Park et al., 2014). Other benefits include the protection from anoikis, as explained before, and to prevent cell aggregations. These beads can be made using different materials, as illustrated in figure 3. To optimize the efficiency of CM production, the composition of the beads and relation to the biotechnological setup must be addressed.

These microcarriers can be designed to include specific ligands for cell binding, resembling specific elements of the ECM. Cells can expand on these microcarriers by binding to such ligands, after which they can expand and differentiate if the appropriate growth factors are present. Such ligands can include ECM protein, such as laminin or vitronectin, which both are shown to promote pluripotent stem cell growth (Lam et al., 2014). Cells can spread from bead to bead through contacts of the beads, or can be spread through enzymatic release of the beads (McKee & Chaudhry, 2017). After cells have expanded on these beads, the concentrated cells can be harvested and subsequently used for further steps within the process of CM production.

5.1 Imbedding microcarriers into CM

A key cost of the process of CM production is the biotechnological process and its associated labor costs (Garrison et al., 2022), making factors within this step another important

consideration. The use of microcarriers is commonly done in large-scale bioreactors, which increases the surface area for cells to grow (‘Unlocking the Secrets of Cell-Cultured Meat Production with Scaffolds’, 2023). Because of the increased surface area for these cells to grow on, the potential yield is higher, so the production of CM is easier to upscale (Bodiou et al., 2020). Microcarriers can be used in conjunction with other (scaffolding) techniques to further increase the efficiency of cell growth. Essentially, microcarriers can be used in a biotechnological setting to create a 3D environment for cells to grow in, which is done in combination with other techniques such as a spinning mechanism within a bioreactor. For example, MSCs, being a potential starting point for CM, have been shown to grow significantly more effectively using such a dynamic setup (Rafiq et al., 2016). An important nuance is that within such a biotechnological setup, the conjunction of microcarriers and a mixing mechanism has been proposed to be key for the success of the use of microcarriers. Next to this apparent successful effect of microcarriers, using the optimal materials to use for microcarriers is still a topic of debate (McKee & Chaudhry, 2017). Biodegradable materials have shown to be viable to construct microcarriers.

Next to the materials used, microcarriers can also be implemented to function in different ways. Microcarriers can be designed to degrade or dissolve during the development of the cells, be embedded into the final product or to be non-degradable, subsequently requiring the separation of the cells and the microcarriers. Though there is a limited amount of data available to say with confidence what way of implementing microcarriers for stem cell expansion is best, making microcarriers in such a way that they are incorporated into the final CM seems to be an effective option (Bodiou et al., 2020). First of all, the inclusion of edible microcarriers allows a greater degree of tailoring of the final product, since the composition of the microcarriers can contribute to this composition. This allows the tailoring of the organoleptic properties of the meat, for example (Bodiou et al., 2020). There are comparative benefits related to the other two methods. The benefits of this option, compared to non-degradable, non-edible microcarriers includes the avoidance of necessitating cell-microcarrier dissociation. For this dissociation additional steps need to be incorporated into the development of CM, such as the enzymatic dissociation of the cells from the microcarriers. Compared to the use of degradable microcarriers, this option could be considered more reliable, since the cells will have a consistent binding target for the duration of the whole process. Cells would need to stay viable till further steps in the process of structuring the cells to constitute tissue which resembles meat.

Such an edible type of microcarrier has been demonstrated to work effectively in a commercial-scale suspension bioreactor. Zernov et al. (2022) have used microcarriers composed of chitosan and collagen biopolymers, with a ratio of 90:10 respectively. These carriers were developed using electrospray, together with the natural crosslinkers TPP and EGCG (Zernov et al., 2022). TPP is added to constitute a 2% concentration and is the main agent responsible for polymerization, while 0.02% EGCG is added to obtain smoother, spherical microcarriers. Such concentrations indicate that variability of components used to generate the microcarriers allows great flexibility of the properties of the generated microcarriers, such as size and shape (figure 5). These microcarriers were shown to be viable using cells from different animal origins, such as primary sheep fibroblasts and primary bovine mesenchymal cells.

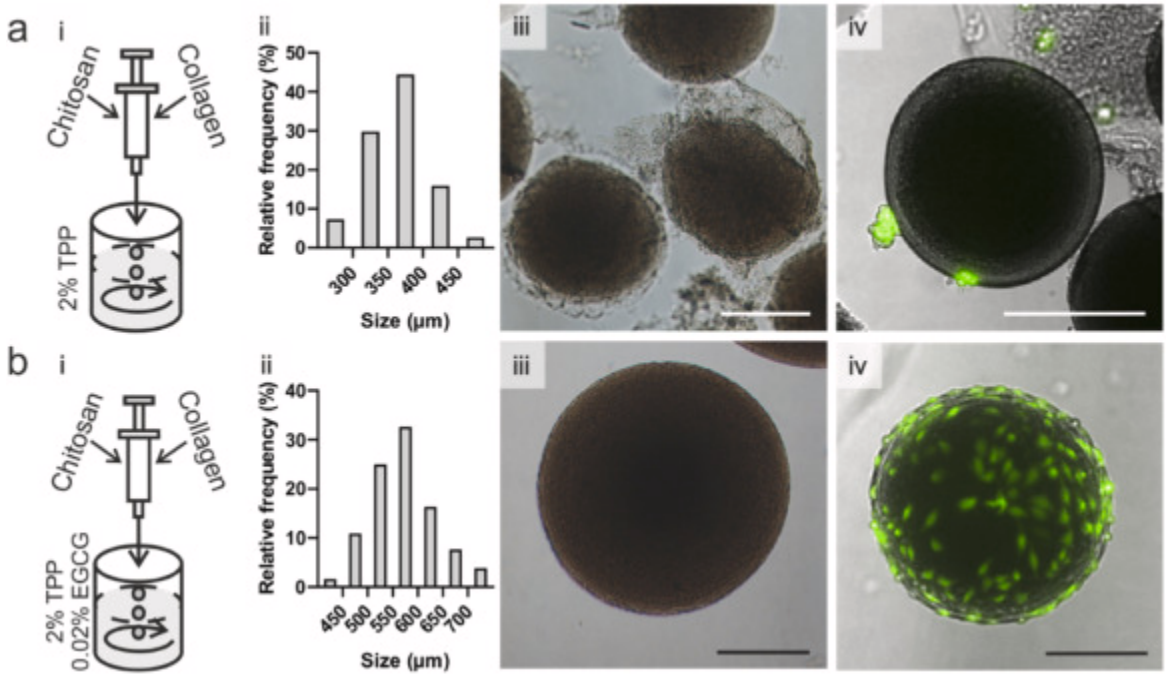

Figure 5, adapted from Zernov et al. (2022). a) chitosan and collagen with 2% TPP. b) chitosan and collagen with 2% TPP and 0.02% EGCG. i) schematic representation of the microcarrier formation process. ii) representation of the size distribution of the microcarriers, addition of EGCG has increased the average bead size. iii) bright field imaging of the respective microcarriers. iv) FDA/PI stained myoblast cells (C2C12) after 2 days of culturing. Green (FDA) signal shows living cells while red (PI) shows dead cells (not abundant).

6.0 Structuring CM

Microcarriers contribute to the effective development of cells which constitute CM, but the question remains of how these cells are properly structured to mimic traditional meat. While the arguments for the use of microcarriers are apparent, which subsequent scaffolding technique to be used to actually structure the cells into a meat-like structure is less apparent and a greater challenge. As mentioned earlier, there are many different applications of scaffolding which attempt to recreate the structure of traditional meat, such as hydrogels, porous scaffolds and 3D printing. Furthermore, there is a need to be selective about the materials used, since the use of plant based materials can greatly reduce the environmental impact the production of this CM has (Seah et al., 2022). Plant based materials align with this vision and can be much less costly than animal alternatives. The scaffold also needs to be biocompatible, biodegradable and needs to have the right scaffold architecture to produce CM which resembles traditional meat. Such qualities link in with the fact that the food needs to be safe according to food regulations.

A combination of multiple biotechnological processes can be combined to obtain the desired mimicking of traditional meat. 3D bioprinting is a scaffolding technique which can be used both in conjunction with the use of microcarriers and hydrogels, another scaffolding technique. This combination has shown to be effective within the domain of tissue engineering (Xu et al., 2019), allowing for a great degree of customizability of the scaffold through the use of a hydrogel which is structured with different materials and growth factors, allowing the development of CM resembling traditional meat.

6.1 Hydrogels

Hydrogels are made of polymers of which a network readily absorbs water due to their hydrophilic nature. The gel is highly customizable and is able to include different macromolecules, such as growth factors. It can be composed of multiple types of gel forming substances, such as fibronectin or gelatin (*Cultivated Meat Scaffolding* | *Deep Dive* | *GFI*, 2021a). Furthermore, these gels allow perfusion of water-soluble molecules and can be highly permeable for oxygen. These qualities make hydrogels an excellent choice for the development of CM cells. Since the composition, i.e. growth factors, stiffness of gel and the permeability of the gel allow for an environment which can be compared to in vivo conditions.

Stiffness and incorporated growth factors allow hydrogels to direct the differentiation of cells. By changing the stiffness, the (stem) cells used can be artificially signaled to differentiate into certain desired cell types, such as fibroblasts, adipocytes and myocytes. MSCs, for example, can be directed towards fat through an elastic hydrogel or towards bone using a stiff hydrogel (*Cultivated Meat Scaffolding* | *Deep Dive* | *GFI*, 2021a). Such elasticity or stiffness can be customized through many different methods, such as changing the degree of polymerization of the material used within the gel. It is possible to generate a hydrogel with spatially differing mechanical environments, thereby facilitating differential differentiation (Freeman & Kelly, 2017). To induce such different micro-environments, 3D printing can be a practical tool, as has been used in (Freeman & Kelly, 2017)). Such 3D printing also allows the creation of pores within the gel, which allows for a greater degree of diffusion of oxygen and the exchange of nutrients and waste materials. Furthermore, growth factors can be incorporated into the hydrogels by mixing them with the precursors (figure 6). Heparin sulfate, for example, can be added to the gel to help sequester these growth factors (*Cultivated Meat Scaffolding* | *Deep Dive* | *GFI*, 2021a). Together with 3D printing, different growth factors can be incorporated into the different micro-environment of the gel, thus further establishing cell types in different regions of the gel.

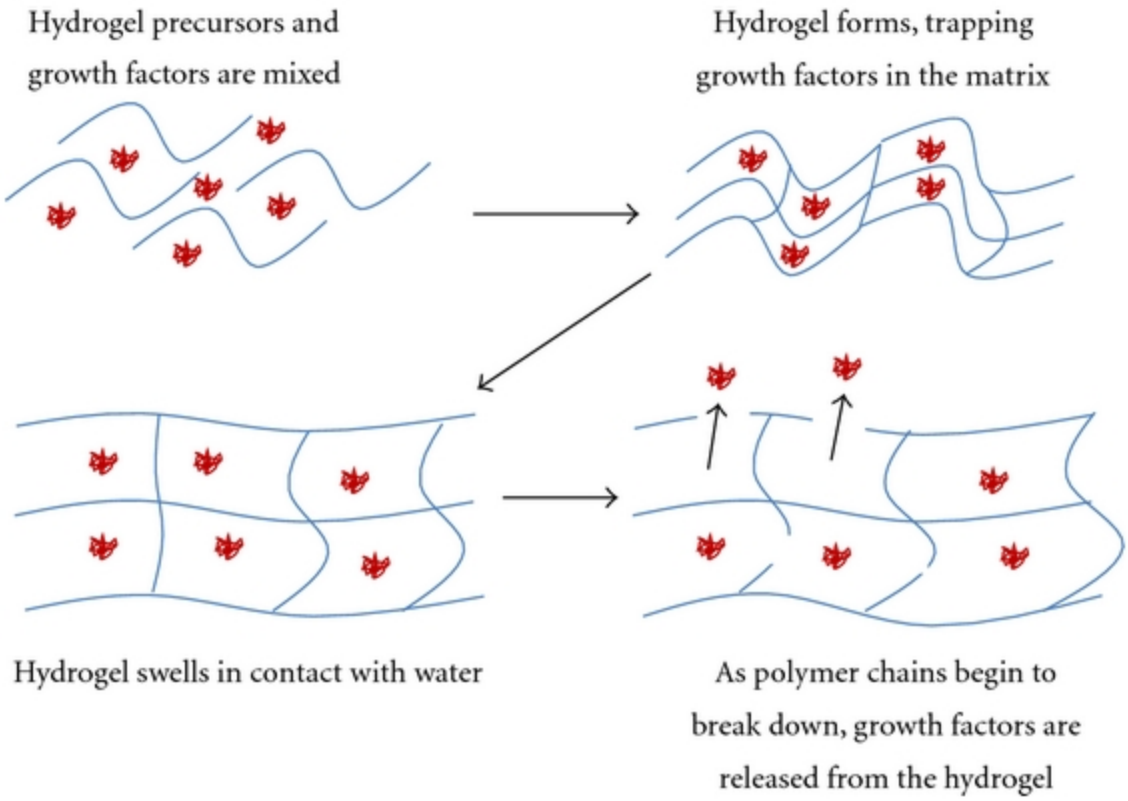

Figure 6, adapted from *Cultivated Meat Scaffolding* | *Deep Dive* | *GFI* (2021). Hydrogels with incorporated growth factors can be created by adding these growth factors to the precursors of the gel. After polymerization these growth factors are trapped within the matrix of the gel. Finally, as cells develop, the gel will break down and the growth factors are released, stimulating the desired differentiation.

6.2 3D bioprinting

There are multiple methods of implementing 3D bioprinting to provide structure for CM, such methods include the use of lasers (e.g. stereolithography), droplets (e.g. Inkjet) or pneumatic driven expulsion of material. The latter, named extrusion 3D printing, is arguably the best fitting 3D bioprinting method to combine with our prior explained methods (*Cultivated Meat Scaffolding* | *Deep Dive* | *GFI*, 2021b).

Extrusion 3D bioprinting is a technique where pre-polymer solutions are injected, using an ‘extruder’, into a gel under the assistance of a computer program. Such a program allows for high customizability of the structure and is one of the most recently advancing methods to structure CM (Tibrewal et al., 2023). The pre-polymer solution can be combined with cultured cells, which results in a combination called ‘bioink’. The advantage is its relative simplicity combined with its ability to print high densities of potentially different types of cells (*Cultivated Meat Scaffolding* | *Deep Dive* | *GFI*, 2021a). The largest drawback is that a viscous material is

needed for the cell material to be inserted into. While using a hydrogel, this disadvantage is thus negated. Essentially, the bioink can be composed of materials which can form a hydrogel, injecting a second hydrogel into a primary hydrogel which gives the initial structure. The process of the physical bioprinting is fairly simple, requiring the aforementioned bioink to start with. The bioink is collected in a chamber, which pressurizes the bioink, making the materials expel out of the extrusion nozzle (figure 7) (Tibrewal et al., 2023). Such a bioink could include multiple cell types, for example fibroblasts and MySCs. Alternatively, multiple nozzles can be used to create microenvironments with different cell types, potentially allowing the option to recreate microenvironments such as that of adipose tissue. Using a computer program to accurately move either the extruder or the target, e.g. a hydrogel, the desired geometric shape including multiple layers can be obtained.

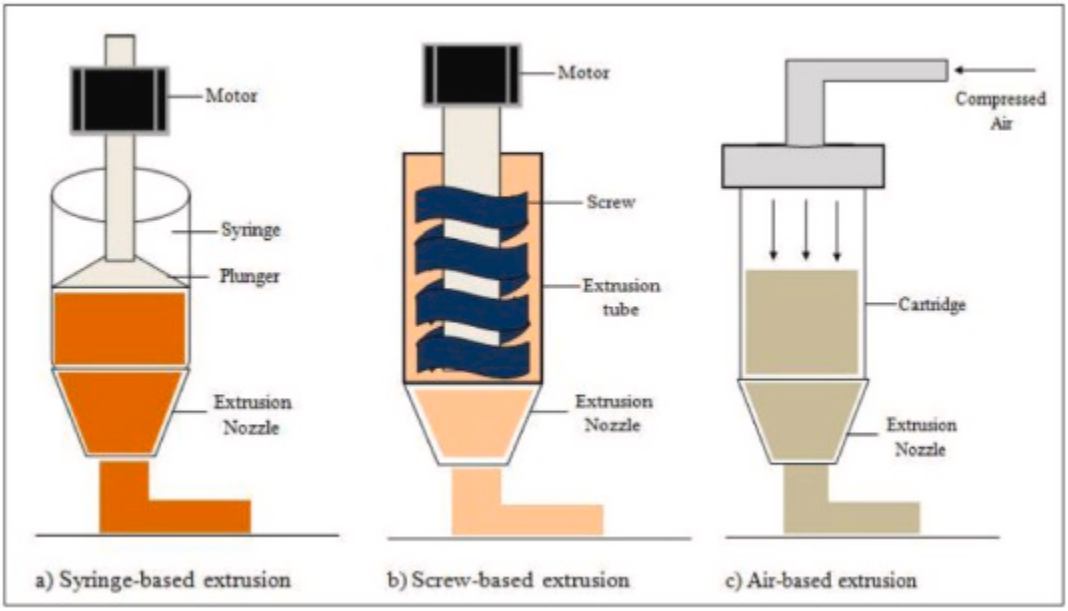

Figure 7, adapted from Tibrewal et al. (2023). Extrusion 3D bioprinting consists of different mechanisms, such as syringe-based, screw-based and air-based extrusion.

Many parameters need to be taken into account when using 3D bioprinting to structure cells for CM, which at the moment are greatly varying within the industry (Tibrewal et al., 2023). Importantly, gelling agents are necessary to assist for efficient expulsion. Such gelling agents can consist of many types of compounds and can be tailored to alter the qualities of the meat, for example reducing the fat content for the meat if desired. For beef a combination of guar gum and animal fat has been used to assist in this expelling of bioink (Dick et al., 2019). Guar gum is a type of hydrocolloid, which are able to form gels and retain water, reducing the shear stress cells are exposed to during extrusion (C. Weller, 2009). Alternatives could include the use of gelatin or polymers such as starch. In this case, a more sustainable alternative could be found to replace the use of animal fat through such alternatives. Other parameters such as nozzle size, extrusion speed and pressure should be taken into account. Such parameters need to be viable together, for larger particle sizes and a larger nozzle the aforementioned hydrocolloids play a vital role for the stability of the bioink and its cells (Tibrewal et al., 2023)

7.0 The ultimate process

Combining all our results, the overall process can be concisely summarized, which are congruent with recent findings (Figure 8). First of all, multiple cell types can be used as a starting point for CM production. Such cells can be taken from live animals or can be retrieved from animals used for meat harvesting. These cells can be cultured with appropriate growth medium, where microcarriers can play a role to significantly enhance the efficiency of proliferation and differentiation. Combined with a biopolymer, such as in the case of hydrogels, the cells can be combined into a viscous liquid called bioink. Through the use of 3D printing, specifically extrusion-based, the cells can be cultured within a printed construct.

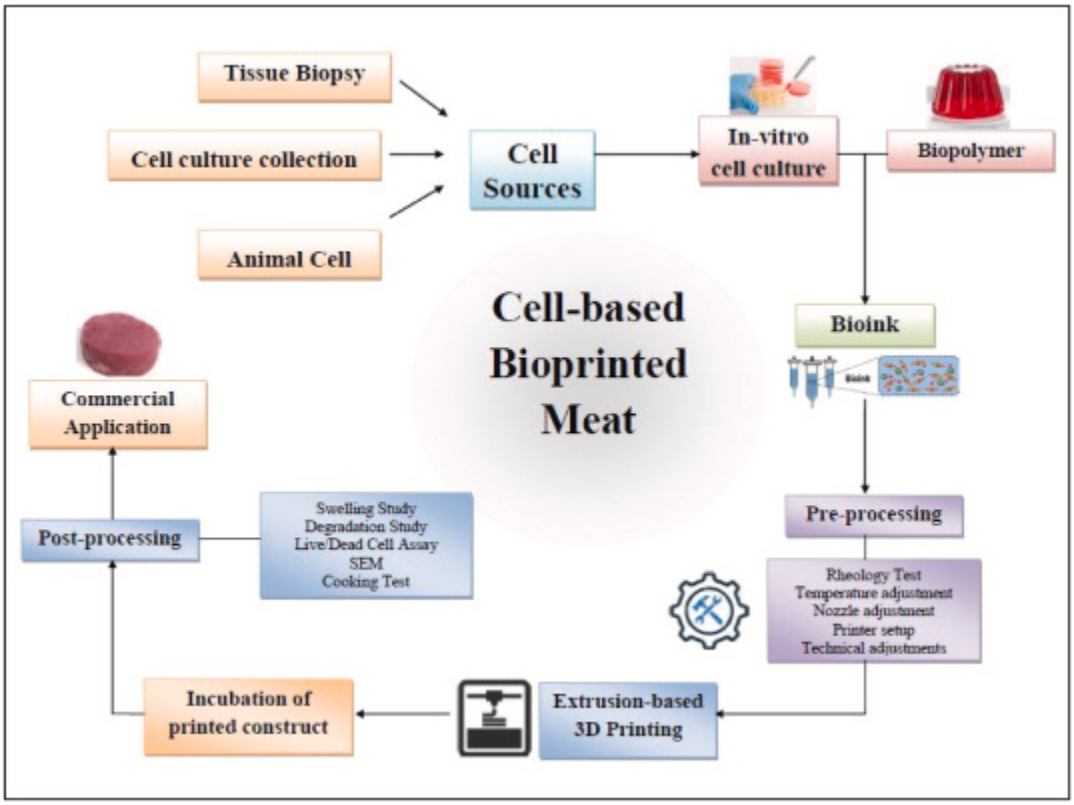

Figure 8, adapted from Tibrewal et al. (2023). The summed up process of commercial CM production starting from cell sources. Steps taken include the in-vitro culturing, combination with biopolymers to form bioink, extrusion-based 3D printing and incubation of the printed construct. Intermediate steps of pre-processing and post-processing show potential adjustments between the steps and can include testing to assure quality and collect data.

8.0 Perspective and conclusion

One aspect of making CM successful is high consumer acceptance, for which scaffolding is key. In this paper we first globally investigate the process of CM production and its scaffoldings step, after which we delve into the details of scaffolding, specifically microcarriers, hydrogels and 3D bioprinting. We find that for the overall process, a co-culture of MySCs combined with fibroblasts to be a viable combination of cells, because of their synergistic ability. We analyze that these cells must be cultured on a scaffold which is biodegradable, biocompatible, must have the right architecture and must be able to be produced efficiently. We investigate the biotechnological field and note the most prominently used methods of microcarriers, porous scaffolds, hydrogels, 3D printing and fiber scaffolds.

Here we find that microcarriers significantly increase the efficiency of cell proliferation and differentiation by drastically increasing surface area and providing anchorage-dependent cells a link. The mechanotransductive element of the scaffold further provides a signal for the integrin-signaling pathway to prevent anoikis from occurring. We discuss the option of using chitosan and collagen, of which the prior is sourced from animals. Even though this option is seemingly efficient, the materials used should both be viable and sustainable. Other more sustainable polymers need to be screened for efficiency and viability. The properties of scaffolding such as microcarriers has often been inspired by the field of TE, which needs to be adapted for CM (Singh et al., 2023).

After cells have been cultured in vitro within a bioreactor, we suggest that hydrogels are an effective next step. Hydrogels are highly customizable, due to easily modified elasticity, permeability and additional ability to include pores. Furthermore, the composition, including growth factors, can be changed. These changes can all be done in specific regions, potentially allowing microenvironments for the development of specific tissue. For hydrogels as well, there are many more considerations which need to be addressed. Because of the relative recency of developments within the field, data is missing to accurately and extensively analyze what materials or other properties are necessary for optimal usage of these technologies.

Lastly, hydrogels are compatible with 3D bioprinting, allowing for an even greater degree of customization. The compatibility of these three technologies forms a solid argument for the usage of the techniques. Due to the broadness of the available techniques within the paper, screening for the appropriate and efficient techniques was one of the most important aspects. In this case, we have chosen to elaborate on 3D bioprinting, due to its apparent compatibility and easy of use. These advantages are real, but lack a tangible element. More studies need to be done to quantify and analyze the efficiency of these (combined) techniques.Through such follow-up studies, parameters of these pipelines can be optimized, contributing to the viability of CM and ultimately reducing the environmental impact humanity has.

All together, while this paper lays a solid framework for the process of CM production, it lacks a certain depth of analysis due to lacking consensus on optimal solutions within this process. Furthermore some aspects of CM production have not been taken into account due to the

broadness of the subject. Aspects of the process, like growth factors, growth medium, cell interactions and differentiation pathways have not been elaborately discussed, which can add to the scientific value of this research. Another key aspect is the regulatory aspect of CM. Large scale commercialization would require further investigation into scaffolds which are congruent with food regulation policies, such as that of the FDA (Levi et al., 2022). While intermittently mentioned, proper economic analysis should be performed to elucidate the optimal techniques for the production of specific CM products. These subjects, together with others such as socio-cultural (Kumar et al., 2021), environmental (Chriki & Hocquette, 2020) and religious aspects need to be elaborated in further research. In this paper we only discuss CM as being a solution for the growing need for sustainable protein sources, which is just one method to produce protein rich food which does not rely heavily on animals (Seah et al., 2022). Future aspects need to be taken into account to investigate all the potential solutions within the field of CM, expanding the options, which can be screened for efficiency and compatibility.

We conclude that CM scaffolds contribute to the viability of the structure of CM products. The combination of a co-culture of MySCs with fibroblasts, in conjunction with microcarriers, hydrogels and 3D printing allows for efficient development of CM with a proper structure. The optimization of parameters for these, or other scaffolding techniques, need to be established to optimize the efficiency of CM, potentially allowing CM to play a great role within the food industry. This large scale implementation of CM could potentially reduce the negative externalities associated with the traditional meat industry.

Afterword

While this research has been one of the most refreshing and interesting literature researches done, balancing the width and depth of the paper has been one of the most difficult aspects. Next to this, the overwhelming amount of data available, which often lacks the conclusive details necessary for analysis, required extensive screening and value judgements. First of all, balancing the width and depth of the paper is necessary, since the total volume is limited and the results should be relevant. The rabbit hole of cultured meat was for us both unexpectedly broad and wide. There are numerous different techniques, materials, parameters and other aspects to be considered. Throughout the screening process, decisions were made to confine the research, which has been difficult. While the amount of different sources give great possibilities, drawing value judgements as to the relevance of information has thus been a great challenge. Realizing this broad versus depth dilemma, for further research purposes a global overview of a subject should better be taken into consideration, before the commitment towards certain aspects of a subject.

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