**Kaunas University of Technology**
Faculty of Chemical Technology

# Investigation of a Zein Fibre Scaffold for Cultivated Meat Production

Master's Final Degree Project

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Supervisor

**Kaunas, 2026**

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## Summary

The global population will tend to grow to almost 10 billion by the year 2050, the demand for animal protein is likely to more than double during the same time span. Traditional animal farming practices, which account for 14 percent of world‘s greenhouse gas emissions, one-third of the world's freshwater consumption, significant deforestation in the tropics, are not able to satisfy the requirement in a sustainable manner. Of the protein alternatives currently being developed, cultivated meat is the only one that can be used to provide the full sensory and structural experience of a conventional whole-cut meat, but it needs a three dimensional structure to enable muscle cells to grow and organise themselves into meat-like tissue. Since this scaffold needs to be eaten as a food product, synthetic polymers, which are employed in the medical tissue engineering industry, are not a possibility, and the only viable option is a plant material.

The thesis aims to explore the potential use of electrospinning to create zein fibrous scaffolds, which is a food-grade prolamin protein from maize, for cultivated meat. A series of seven experimental sets was systematically investigated to observe the influence of solvent ratio, rate of flow, voltage, the distance between needle-to-collector, additive composition, and also zein concentration on the fibre morphology, fibre diameter, and pore size. The 70:30 ratio of ethanol:water was determined to be optimal, resulting in stable ribbon fibres without beads and with an open porous structure. Glucose alone resulted in the most compacted fibres and glycerol-glucose combination gave the best fibre diameter and pore size. Chemical integrity of zein protein matrix was confirmed by FTIR in all conditions, while the XRD showed a major amorphous structure which is desirable for an edible scaffold. The optimized conditions elaborated here could serve as a reproducible platform for scaffold fabrication for future cell seeding and tissue formation studies in a food-grade medium.

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## Santrauka

Prognozuojama, kad iki 2050 metų pasaulio gyventojų skaičius priartės prie 10 milijardų, o gyvūninės kilmės baltymų paklausa per tą patį laikotarpį daugiau nei padvigubės. Tradiciniai gyvulininkystės metodai, kurie sudaro apie 14 % pasaulinių šiltnamio efektą sukeliančių dujų emisijų, sunaudoja trečdalį pasaulio gėlo vandens išteklių ir reikšmingai prisideda prie tropinių miškų naikinimo, negali tvariai patenkinti augančio poreikio. Iš šiuo metu kuriamų alternatyvių baltymų šaltinių kultivuota mėsa yra vienintelė technologija, galinti užtikrinti visapusišką tradicinės vientisos mėsos juslinę ir struktūrinę patirtį. Tačiau tam būtina trimatė struktūra, leidžianti raumenų ląstelėms augti ir organizuotis į mėsą primenantį audinį. Kadangi šis karkasas turi būti vartojamas kaip maisto produktas, medicininėje audinių inžinerijoje naudojami sintetiniai polimerai nėra tinkami, todėl vienintele perspektyvia alternatyva išlieka augalinės kilmės medžiagos.

Šio darbo tikslas buvo ištirti elektroverpimo technologijos potencialą kuriant kultivuotai mėsai skirtus pluoštinius karkasus iš zeino – maistinės paskirties kukurūzų prolamino baltymo. Siekiant įvertinti tirpiklio santykio, srauto greičio, įtampos, atstumo tarp adatos ir kolektoriaus, priedų sudėties bei zeino koncentracijos poveikį pluoštų morfologijai, pluoštų skersmeniui ir porų dydžiui, buvo sistemingai atliktos septynios eksperimentų serijos. Nustatyta, kad optimalus etanolio ir vandens santykis yra 70:30, nes tokiomis sąlygomis susidaro stabilūs juostiniai pluoštai be defektinių lašelių (beadų), pasižymintys atvira porėta struktūra. Glikozės naudojimas vienos priedo forma lėmė labiausiai sutankintą pluoštų struktūrą, o glicerolio ir glikozės derinys užtikrino palankiausius pluoštų skersmens ir porų dydžio rodiklius. FTIR analizė patvirtino, kad visomis tirtomis sąlygomis buvo išsaugotas zeino baltyminės matricos cheminis vientisumas, o rentgeno difrakcijos (XRD) tyrimai parodė vyraujančią amorfinę struktūrą, kuri yra pageidautina valgomiems karkasams. Šiame darbe nustatytos optimizuotos elektroverpimo sąlygos gali būti naudojamos kaip atkuriama platforma maistinės paskirties karkasų gamybai ir sudaryti pagrindą būsimiems ląstelių sėjimo bei audinių formavimo tyrimams maistinės terpės sąlygomis.

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## Abbreviations:

| | |
|---|---|
| iPSC | Induced pluripotent stem cell |
| kPa | Kilopascal |
| kV | Kilovolt |
| LCA | Life cycle assessment |
| MHC | Myosin heavy chain |
| mL/h | Millilitres per hour |
| MOH | Ministry of Health (Israel) |
| MSC | Mesenchymal stem cell |
| MuSC | Muscle satellite cell |
| NaCl | Sodium chloride |
| NRG1 | Neuregulin 1 |
| OTR | Oxygen transfer rate |
| PDCAAS | Protein Digestibility-Corrected Amino Acid Score |
| PEO | Poly(ethylene oxide) |
| pH | Potential of hydrogen (measure of acidity/alkalinity) |
| RGD | Arginine–glycine–aspartate (cell adhesion peptide sequence) |
| RH | Relative humidity |
| RMB | Rocking-motion bioreactor |
| RNA | Ribonucleic acid |
| SC | Satellite cell |
| SFA | Singapore Food Agency |
| SPI | Soy protein isolate |
| STR | Stirred-tank bioreactor |
| TERT | Telomerase reverse transcriptase |
| TFF | Tangential flow filtration |
| TGFβ1 | Transforming growth factor beta 1 |
| TSP | Textured soy protein |
| USDA | United States Department of Agriculture |
| USDA-FSIS | United States Department of Agriculture – Food Safety and Inspection Service |
| µm | Micrometre |
| 2θ | Diffraction angle in X-ray diffraction |

# Introduction

There is a tipping point for the world food system. By the year 2050, the world will have about 9.7 billion people, and by about 100% more demand for animal-based protein will be placed on it. But the environmental costs of current animal protein consumption are already unsustainable. The livestock sector accounts for about 14 percent of all human induced greenhouse gas emissions, uses almost one third of the global freshwater footprint in livestock production and is one of the main causes of deforestation in tropical areas, especially in Latin America, where 65% of forest losses are attributed to the cultivation of soya beans and the production of livestock feed [1-3]. In 2023, 733 million people would faced starvation, and more than 2.33 billion people are speculated to face moderate or severe food insecurity, while traditional agriculture has not solved the overproduction and under-distribution problem.

Cultivated meat is considered  a technologically fascinating protein substitute, both from a scientific and commercial perspective. Unlike plant-based alternatives that can't fully match the sensory and structural richness of whole-cut meat, or the cultivated meat is grown directly from animal cells, potentially creating a more environment-friendly means to produce real meat [4]. Since the breakthrough cultured beef demonstration by Mark Post in 2013, more than USD 3bn has been invested in the sector and regulatory approvals are granted in countries like Singapore in 2020, in 2023 the United States was accepted, Israel and Australia in 2024 [5, 6]. In spite of this, there remain significant unsolved issues to address when scaling up cell culture, cost of media, and most importantly, when targeting structured meat products, the development of scaffolding systems that are biocompatible, structurally sound, and safe to eat by humans [7, 8]. One enabling challenge for structured cultivated meat is scaffolding. Muscle cells will live, grow and develop if they are provided with a physical substrate. To provide cells with nutrients and oxygen throughout the thickness of the construct, any construct thicker than 200 μm needs to be porous and perfusable [8].

Perhaps more crucially, the scaffolds for cultured meat cannot be synthetic polymers as is the focus of most medical research in scaffold development, but must be digestible as part of their food product. In line with the overall sustainability concept of the technology, a polysaccharide based on plant resources like cellulose, alginate, starch and a protein like soy, pea or zein is a highly promising food grade, biocompatible and scalable alternative [9, 10]. In the plant protein fraction, zein, the prolamin storage protein of maize, is especially suitable for use in the fabrication of scaffolds using electrospinning: it is soluble in aqueous ethanol; solidifies under high-voltage electric field conditions to form continuous fibres; is classified as a food additive by the FDA, and has been proven to have biocompatibility with mammalian cell culture systems [11, 12].

## Aim and objectives

### Aim:

The aim of this study is to develop electrospun zein-based fibrous scaffolds for cultivated meat applications and to investigate how solution composition and electrospinning process parameters affect fibre formation, scaffold morphology, chemical integrity and structural properties.

**Objectives:**

1. To develop a literature based research plan for the fabrication of edible plant protein fibrous scaffolds for cultivated meat production, with emphasis on zein.

2. To prepare zein electrospinning solutions containing food compatible solvent systems and additives, and to investigate the influence of ethanol to water ratio, zein concentration, glycerol, glucose, PEO and NaCl on solution electrospinnability.

3. To fabricate zein fibrous scaffolds by electrospinning and investigate the effect of key process parameters, including flow rate, applied voltage and needle to collector distance, on process stability and fibre formation.

4. To characterize the obtained zein scaffolds in terms of fibre morphology, fibre diameter, pore architecture, chemical composition and structural organization using optical microscopy, SEM, FTIR and XRD, and to identify the most suitable fabrication conditions for further cultivated meat scaffold studies.

# 1. Literature Review

## 1.1. Global protein demand and the challenge of feeding the growing populations

Protein is a vital component of the functional and structural sustenance of all biological systems of the human body. Reasonable world protein demand is not only a challenge for agriculture but also an issue to question the food security, well-being of the population and the sustainable development. It is presumed that by the close of the year 2050, the number of human beings would exceed nine billion, which will once again add to the demand for protein excess [13]. People's eating habits have changed as a result of the low- and middle-income countries' economies and population growth.

It is projected that the necessity of protein is striking, as it is evident that the data collected from an analysis of 50-plus global food security forecasts. It claims that the worldwide protein demand would increase from 35% to 56% over the period 2010-2050 [14]. Another analysis shows that the total food demand would be growing from 50% to 60% by the year 2050, and particularly animal-based protein is likely to double [15].

### 1.1.1. Environmental cost of standard animal farming

The environmental footprint of the current livestock production methods puts a hard limit on the ability of the global food system to satisfy this demand. Animal husbandry has extraordinary, and in most instances, unsustainable pressures on land, water and the world climate. Regarding greenhouse gas (GHG) emissions, the livestock industry is among the most important sources of anthropogenic climatic change. According to FAO historical data, anthropogenic GHG emissions from livestock supply chains are 7.1 gigatonnes of CO2 equivalent each year, which represents 14.5% of the global anthropogenic GHG emissions. The three main sources of emissions are manure decomposition (10%), enteric fermentation (mostly from ruminants) (39%) and feed production and processing (45% of the total) [1].

Animal farming is also very demanding in terms of water consumption. Agriculture contributes to 92 percent of the freshwater foot print of the human race and nearly a third of this is associated with animal products [2].

### 1.1.2. Socioeconomic viewpoints and food security

Even in high income countries, large parts of the world are protein deficient and protein excess is causing environmental problems. Animal-based products made on the land (meat, milk and eggs) support the daily food requirement and food security of approximately 1.3 billion people, including 930 million in Africa and South Asia [3]. It is hence a paramount equity factor that alternative protein solutions be nutritionally sufficient, affordable, and accessible to low- and middle-income groups.

### 1.1.3. Cultivated meat as an alternative

The combination of these forces: demographic, environmental, and those related to public health, and ethical, has created significant scientific, commercial and policy interest in so called alternative proteins: protein sources that are capable of satisfying human nutritional requirements and functioning at a significantly reduced environmental cost than traditional animal agriculture [7].

It is estimated that the alternative protein market will be USD 17.97 billion in 2024 and USD 50.45 billion in 2035, as the current and potential investors and consumers are eager to find a solution to the problem of decoupling protein production and its environmental and ethical impact . Protein-derived strategies may be generally categorized into the following: plant proteins (soy, pea, wheat, etc.), fermentation proteins (produced by microorganisms or fungi), insect proteins, algae and microalgae, and cell-cultured or cultured meat - the production of animal muscle tissue directly using cells, without the growth and slaughter of the entire animal [4, 16].

The literature review dwells specifically on the cultivated meat, and on the biological and material engineering problems that need to be surmounted to make it a commercially viable enterprise, with particular emphasis to the contribution of scaffold technologies as an enabling basis to showcase structured meat products.

### 1.2. A way to resolve the world wide protein challenge

The world wide protein deficit and the consequences of its environmental impacts require a multi-dimensional approach through technological innovation, agricultural system restructuring, behavioural change, and the provision of enabling policy frameworks. It will not do to implement a single intervention, but a portfolio approach will be needed that can both mitigate the environmental footprint of the current food systems and scale new production methods to commercial viability [17]. This chapter explores the key solution categories and places the evolution of alternative proteins and cultured meat.

#### 1.2.1. Classification of alternative proteins

Alternative proteins and innovations in food processing technologies are crucial to solve the complex challenges of global protein demand growth, climate change and resource limitation. Alternative proteins refer to meals or ingredients rich in protein with sensory, nutritional and functional properties comparable to those found in dietetic animal protein. This review includes plant proteins (soy, pea, wheat gluten, pulses, and oilseeds), microbial proteins and fermentation proteins (mycoprotein, microalgae, and single-cell proteins), as well as insect proteins and cultured (cell-based) meat, and multi-protein hybrid systems [18].

#### 1.2.2. Plant-based protein

The most developed and outstanding types of protein substitutes are plant protein. Due of the global movement in consumer tastes for more sustainable and healthful food options, plant-based meat substitutes—which are usually manufactured from soy, peas, wheat, rice, and mung beans—have recently gained prominence [19]. Most dietary protein is already plant-based, with 57% of all protein consumed globally coming from plant sources and total alternative protein (including plant-based) consumption estimates 13 million metric tonnes in 2020 - about 2% market of the animal protein [14]. These proteins are isolated, concentrated or extracted from their host crops and then further processed - typically via extrusion - to create fibrous products that mimics the texture, feel and look of meat products [17].

For both humans and plants, pulses are an essential source of nutrients and protein. Pulses are an important source of protein in many parts of the world and for some individuals, the primary source of protein. Indeed, pulses are crucial in addressing the issue of protein-energy malnutrition in the emerging and impoverished globe. The pulse crop cowpea (*Vigna unguiculata*) is present in East and West Africa, for instance. It is rich in nutrients with carbohydrate content ranging from 60 to 65%, crude protein content between 21 and 25%, lipids content 1 to 1.5%, moisture 10% and ash 2.5–4%. Some pulses - such as lupin and soy beans - are reported to have 45-50% protein, but chickpeas are an exception with 4-5% fat. There are trace amounts of protein in the seed coat, and cotyledon and embryonic axis of the pulse seed. Cotyledons (which constitute the bulk of the pulse seed) are the main source of protein. The protein concentration in pulse proteins, like cereals, is highly variable, depending on cultural practices, environment and genetics. Concerns have been raised that a protein imbalance leads to malnutrition in vegans (or in diets that have plant proteins as the primary source of protein) [20]. This is because, unlike animal proteins, plant proteins may not have all the essential amino acids [21].

Soy protein isolate is one of the best balanced plant proteins in terms of nutrition (Protein Digestibility-Corrected Amino Acid Score (PDCAAS) of 1.0 (equivalent to animal protein) because it contains all amino acids [20].

### 1.2.3. Insect-based protein sources

Because of their high feed conversion ratio, minimal greenhouse gas emission, and ability to add value to organic wastes, insect proteins have garnered a lot of interest as alternative proteins [22]. Some of the most popular insects raised include mealworms, crickets, and black soldier fly larvae, which are claimed to have a relatively high protein, fat, mineral, and vitamin content. Further, they can be ground up to produce flour and protein concentrates that can be incorporated into diverse foods [23].

In particular, insects like black army fly, house fly, beetle, mealworms, silkworms, earthworms, crickets and grasshoppers are being promoted because they have been recognized as a good and sustainable source of animal protein for chicken feed. Insect meals are nutritionally adequate for poultry and contain all essential amino acids. These are well-digested and are accepted by the birds. In addition, they are rich in antimicrobials and other bioactive molecules, which enhance human health [24].

Insects are rich in minerals such as iron and zinc, and vitamin B complex (B1, B2, B3) as well as important amino acids. The fatty acid composition of their product also has value, as the ratio of the three types of fat (SFA, MUFA and PUFA) falls within the recommended range for health [25].

Furthermore, compared to other animal foods, insects have a significant amount of dietary fibre, and this makes insects a well-balanced food. The potential of insects as a valuable food has been acknowledged. Insects have been proposed by the World Health Organisation as an adequate food to satisfy the protein demand of malnourished people. It also has a good nutritional impact in HIV positive people, who need to improve their nutritional well-being to prevent immunological deterioration [25].

### 1.2.4. Fermentation-derived proteins

There are two variants in biotechnology based fermentation, the biomass fermentation and precision fermentation. Microbial biomass is the result of direct fermentation of biomass, which is a direct fermentation based protein manufacturing method. A type of edible microbial biomass produced using microorganisms including bacteria, fungus, archaea, yeasts or algae is called as single cell protein (SCP). There is no need for cultivable land or fresh water and the process of SCP manufacturing is continuous, which can accommodate agricultural wastes and byproducts. The first and most widely known case of biomass fermentation is the production of mycoprotein.The most well known case of biomass fermentation is continuous fermentation of the filamentous fungus *Fusarium venenatum.* Mycoprotein has a protein content of around 45% (dry matter), a good amino acid profile. It is a rich source of dietary fibre from the fungal cell walls, imparting a naturally fibrous meat-like texture, thereby avoiding the need for costly mechanical treatments [26].

On the other hand, precision fermentation involves programmed microorganisms to create functional proteins (like bovine whey proteins and caseins) that are then extracted from the microbial biomass and in turn used as protein ingredients for food. Precision fermentation produces animal ingredient proteins in microbes, and is purely independent from outdoor farming in terms of food production. It comes with an added benefit of removing the risks associated with the market dynamics that drive current energy and food markets. Examples include Perfect Day (animal-free whey protein in Trichoderma reesei) and Remilk (recombinant milk proteins in yeast) [27].

### 1.2.5. Algae and microalgae proteins

Algae (seaweed and microalgae) are a good protein source with unique nutritional properties and a small land footprint. Microalgae generally contain 35-70% protein (dry biomass), 10-30% carbohydrates, and 5-28% lipids, depending on the microalgae species, cultivation conditions such as light, temperature and pH, and nutrients) and the growth stage of the culture [28].

The best-known microalgae used for human food are Spirulina (*Arthrospira platensis*) and *Chlorella vulgaris,* which are approved as food supplements and ingredients in the European Union and the US. Algae is also better than many other types of plant proteins as they contain a significant amount of the necessary amino acids, carbs, lipids, and minerals. They also contain bioactive chemicals with anti-hypertensive (blood pressure reducing) and antioxidant (free radical scavenging) properties [29].

### 1.2.6. Comparative summary and positioning of cultured meat

A recent review compared various alternative protein production options under four key parameters (environmental impacts, scalability, consumer acceptance, and animal welfare) and found that plant based meats were the most promising due to the existing infrastructure, high consumer acceptance, and adequate environmental benefits; cultured meat, less technically and economically viable, was showing potential; single cell proteins, with unknown scalability, were also promising; and products derived from insects, were not yet ready for large-scale production [30]. This reveals that while not all alternative protein sources are created equal, they have different, but potentially complementary, roles and applications. Importantly, plant and fermented food proteins can be possibly used as alternate for meat for minced and processed meat products, but are yet to match the sensory and nutritional quality and textural structure of the most valuable meat products, whole muscles. This is where cultured meat is set to target and the focus of our current review [31].

## 1.3. Cultured Meat

### 1.3.1. Cultured Meat: from concept to strategy

Cultured meat has various terminology in scientific literature, such as cultivated meat, sometimes as cell-based meat, also as in-vitro meat. It is defined as animal muscle and fat tissues that are produced in-vitro growth and differentiation of animal cells, without having to raise and slaughter animals. Cultured meat harnesses cultured animal cells to produce meat or meat proteins and fat tissue for human consumption, based on the principle of mammalian tissue development and regeneration [32].

The term artificial meat is commonly employed in the media but it is typically not popular among the scientific and industry communities due to the harm that a laboratory-scale and experimental setting would cause, which is not consistent with the purpose of industrial-scale production. Terminology choice has regulatory implications for classification and labelling, and has been a subject of intense policy debate, especially in the United States, the European Union and also in Singapore [33].

### 1.3.2. Concept of developing Cultivated Meat

Cultivated meat has been an idea for many decades before our current understanding of cell biology. It has long been speculated about by British politician Winston Churchill in his essay Fifty Years Hence (1931). His visionary statement was written at a time when Alexis Carrel's tissue culture of chicken heart muscle, begun in 1912, was already growing in the laboratory at the Rockefeller Institute for nearly 20 years, and demonstrated for the first time the possibility of keeping animal cells alive out of the body for extended periods of time [5].

The Dutch scientist Willem van Eelen, who had himself suffered hunger as a prisoner of war, independently envisioned meat production using the use of cell culture, in 1950s; and in 1997, he put the first general patent on meat production using cell culture, and led a team, including professor Mark Post, to investigate the potential of cultivated meat products. NASA scientists created one of the earliest proofs of concept of seafood cultivation in the form of a tiny strip of goldfish muscle tissue in March 2002, in research to supply alternative food to astronauts on long space missions. Moreover, the design of an edible cultivated beef patty by Dr. Mark Post made the concept of cultivated meat popular. The first country to authorise the cultivated chicken meat sale was by Eat Just is Singapore that recently did it. Recently, the US followed the example of Singapore, receiving pre-market approval of the produced chicken meat of the Eat Just. [5].

### 1.3.3. Scaling up and Investment on Cultivated Meat

Over $3 billion in funding has been invested in privately-owned companies focused primarily on cultured meat since 2013. Mosa Meat (Netherlands - bovine beef, established by Post himself), UPSIDE Foods (USA - poultry and beef), GOOD Meat / Eat Just (USA / Singapore - cultivated chicken, the first product to gain approval in the world), Aleph Farms (Israel - beef steaks, including a thin-cut steak prototype), SuperMeat (Israel - chicken), BlueNalu (USA - cultivated seafood), and Vow (Australia - cultivated quail, now in manufacturing in a 15 major conventional food industry companies such as JBS, Tyson, Cargill, Nestlé, and Danone have maden several provisions such as investments, partnerships, acquisitions, and R&D agreements in the cultivated meat area [6].

### 1.3.4. Production strategies: right from minced to structured products

One of the strategic distinctions in the cultured meat sector is the two categories of products minced or unstructured products and structured or whole-cut products which are considerably different in terms of the technical complexity and technologies they require.

Minced and unstructured foods such as sausages, meatballs, patties and nuggets represent the short term commercial route. These products incorporate existing food production methods (extrusion, molding, mixing with plant-based binders) to process, texture and shape cultured cell biomass that has been fermented into a paste or slurry. These products simply need sufficient cell biomass of the appropriate composition; they do not need intricate scaffolding, vascularization, and three dimensional tissue structure [34].

The long-term strategic objective of the field and the most consumer-valued and technical market segment are structured and whole-cut goods, i.e., steaks, fillets, and chops. Three-dimensional scaffolding, multi-cell-type co-culture, nutrient perfusion, mechanical stimulation are all necessary to replicate the hierarchical organisation of whole muscle tissue, parallel myofibre bundles, intramuscular fat (marbling), connective tissue, and blood vessel architecture. Creating structured cultured meat with a texture and taste reminiscent of traditional chicken breasts, beefsteaks or fish fillets is a very difficult endeavor, given that such are still very new technologies that must find a solution to vascularisation, scaffold architecture and cell density that are not currently available at commercial scale [7].

## 1.4. Technologies used to Produce Cultivated Meat

### 1.4.1. Production Process of Cultivated Meat

The cultivated meat is made by a sequence of bioprocesses using a small animal biopsy to produce food-grade tissue. The cultivated meat production using satellite cells can be summarized in 4 sequential actions: Satellite cell isolation of a muscle biopsy, isolation cell expansion, formation of myotubes and mature muscle fibres, assembly of the formed myotubes into a structured food product using food processing methods such as mixing and moulding [35].

The nature of the biological and engineering problems involved in each stage is unique, and the efficiency with which each stage is performed has an effect on the cost and quality of the final product. It has been studied that to produce 10-100 kg of cultured meat, $10^{12}$ and $10^{13}$ cells are utilized [36].

Measurements, orders of magnitude larger than typical cell biology laboratory equipment, are required, and special, food-grade bioprocess infrastructures are required. These four main technologies include source of cells, culture media, scaffolds, and bioreactor systems which collaborate to produce cultured meat [7].

### 1.4.2. Isolating the cells and storing

It begins with a minimally invasive muscle biopsy of a donor animal (usually under local anaesthesia) in which target cells (usually muscle satellite cells or mesenchymal stem cells) are isolated by enzyme digestion of the sample and then selected or sorted. They then grow these early cells in small scale culture vessels and assay them in order to identify, purify and sterilize after which they are frozen and stored in a functional cell bank. An important quality control measure which is very crucial for a commercial scale, is cell banking. It ensures uniformity between manufacturing batches and does not require repeated animal biopsies at a commercial scale [8, 37, 38].

Short replicative life of the primordial cells is consider as one of the major challemge. Normal diploid cells can survive a limited number of population doublings, called the Hayflick limit, and go into senescence, no longer able to reproduce. Researchers have studied cell immortalization method, including telomerase reverse transcriptase (TERT) overexpression and expression of certain oncogenes to create cells capable of endlessly multiplying. There are several steps that can be followed in order to make the expansion stage more efficient, which should work on the level of optimization of the environmental conditions, as well as direct control over the genetic and molecular factors influencing the regulation of the cell division. However, genetic modification presents regulatory and consumer acceptance problems to a large degree in countries such as the European Union where genetically modified foodstuffs (GMOs) are already highly regulated [8].

### 1.4.3. Expansion of cells

After a sufficient cell bank has been established, cells enter the proliferation. In this stage cells are induced to divide rapidly to produce the very large cell numbers needed to produce food. A small sample of cells is prepared to grow into large biomasses in the cell expansion stage, then cell differentiation and tissue maturation, such as formation of muscle fibres and lipid accumulation [39].

In proliferation, cells are grown in a defined growth medium with growth factors (most importantly fibroblast growth factor 2 and insulin-like growth factor 1, which prevent differentiation and induce rapid division [7].

In the case of adherent cell types like myoblasts and satellite cells, it is not directly possible to use a physical attachment substrate to suspend cell culture in standard bioreactors. In order to solve this issue, microcarriers were developed. Microcarriers are microspheres (with a diameter of approximately 100-300 µm) which are coated with cells to give them a larger surface area for cell growth. Micro porous microcarriers are manufactured by a range of materials such as dextran, gelatin, collagen, and cellulose and used with both attached and floating cells. The microcarriers are then mixed with the culture medium and cells in a 3D bioreactor to create a microcarrier-cell complex. Microcarriers are thereby a bridging technology that lies between the 2D culture environment of typical cell biology, and the fully 3D environment of a scaffold-based culture necessary to produce structured meat. One of the major difficulties related to the microcarrier culture method is that shear stress created in the continuous mixing process involving the cell-microcarrier complex inhibits cell activity and proliferation [40].

Balancing the optimisation of adequate mixing that is mandatory to deliver oxygen and nutrients and tolerable hydrodynamic shear has become a major bioprocess engineering concerns during the expansion phase. Computational fluid dynamics (CFD) modelling has become one of the major tools used to predict the shear stress distributions in bioreactor vessels and to guide the design of impeller and vessel geometry to minimise the damage of cells and to maintain mass transfer efficiency [41].

### 1.4.4. Phases of differentiation and maturation

The mitotic growth factors in the culture media are eliminated to prevent the proliferation phase, and replaced with a differentiation medium when sufficient cell populations have been obtained, typically at cell densities of $10^6$ to $10^7$ cells per mm in a bioreactor. This leads to cell cycle arrest in myogenic cells followed by cell fusion, myotube formation and gradual production of contractile proteins such as titin and myosin heavy chain (MHC), which are properties of adult, striated muscle fibres [8].

Mechanical stimulation, which is regulated by signalling channels, regulates the growth and differentiation of muscle stem cells and adipogenic progenitor cells. Mechanically induced stress of bioreactors in muscle cells can be used to control the development of myoblasts converting into mature myotubes and myofibres, which determine the outcomes such as texture of cultured meat. Differentiation is conducted on 3D scaffolds to facilitate the support of structured and whole-cut products instead of being in suspension or on flat microcarriers. Expanded cells differentiates on 3D structured scaffolds, after it is done, if entire meat cuts are required, it is extracted out by 3D printing technology [36].

### 1.4.5. Bioreactors used to produce Cultivated Meat

Bioreactors are the most crucial elements of the cultured meat production process as they are enclosed and controlled spaces with constant monitoring and regulation of temperature, pH, dissolved oxygen (DO), CO2 concentration, and nutrition delivery to maintain cell viability and facilitate effective growth. The most common types are stirred and airlift/bubble column bioreactors, which are imperative to achieve cell adhesion and multiplication. The demand to produce cultivated meat technology has become the need of the hour, and it tends rise rapidly and steadily. The cultivation of the cells is performed in a range of bioreactors both traditional and new with their peculiarities [41].

The most important bioreactor parameters that need to be controlled are: temperature (37C for mammalian cells), pH (7.274), dissolved oxygen (2050% saturation), glucose concentration, and lactate accumulation (toxic metabolic by-product) [8].

The industries standard for large-scale growth of animal cells is the stirred tank bioreactor (STR), which is adapted from the pharmaceutical and biopharmaceutical industries. Stirred-tank bioreactors are thought to be the most common type of bioreactor for producing cultured meat. They have proven to be valuable tools to differentiate and culture cells in 25 mL or 100 mL STR, with or without microcarriers [36].

Transfer rates of oxygen through sparging, well-developed scale-up techniques, well-characterized hydrodynamics, and the ability to operate on either batch or continuous modes of operation are all advantages of STRs at industrial scale. To achieve cell densities and cell volumes of scale suitable to food-scale cultured meat, scale-up of bioreactor vessels will likely be required to increase in scale, similar to bioreactor-scale animal cell cultures. The revolving impeller creates hydrodynamic shear stress, which at high agitation rates can harm or lyse delicate anchorage-dependent cells is considered to be the major drawback of STRs for cultured meat [41].

Cells are planted onto a pervious concave fibre matrix in concave fibre bioreactors, which enables the cells to stick to the face without gumming the medium's rotation. It's believed that this system is best suited to be used in cells with a high metabolic rate, at low mechanical stress and easier access to nutrients. Its crucial sins are low oxygenation, limited scalability, relative complexity, and reliance on concave fibres. The parameters like limited oxygenation, lower scalability, relative complexity, and reliance on concave fibres are its major excrescencies [42]. One of the research indicates that hollow fibre bioreactors support centimetre-scale perfusable cultured meat structures that consists of close and well aligned muscle fibres [43].

Wave bioreactors, are bioreactors that use a rocking platform as a system of mixing in a single disposable use plastic bag with low shear. It has created a 1,000 L scale rocking stir bioreactor and a 2,000 L scale rocking stir bioreactor for cell culture. In terms of scalability, sterility, and functional inflexibility, these bags' large capacity is relatively profitable. By doing down with the need for a thorough washing, they also lower the possibility ofcross-contamination and streamline the bioprocessing process [36].

The most promising, and developed type of large-scale cultured meat production that would be cost-competitive is perfusion bioreactors. Perfusion mode In the perfusion mode, fresh medium is continually fed to it, whilst spent medium, which has been emptied of nutrients and enriched with metabolic waste, is continuously removed through a cell-retention system such as a tangential flow filtration (TFF) membrane. In a pioneering study published in Nature Food (2024), it was shown how the tangential flow filtration can be used to further produce cultured meat. It led to biomass growth to 130 billion cells per litre, which is equivalent to 43% weight per volume yields. The constant operation was possible to last more than 20 days to realize biomass harvests daily. Using this perfusion technology, a techno-economic analysis of a hypothetical 50,000 L production facility utilizing this type of perfusion revealed that the cost of cultured chicken could also decrease to USD 6.20 per pound. This discovery is a significant breakthrough because it has demonstrated, after many years, that continuous production can make cultured beef prices a price range within the spectrum of high quality conventional goods in the market [44].

### 1.4.6. Modes of bioreactor operations

The operation mode (batch, fed-batch or continuous) has a profound influence on the media consumption, bioreactor productivity and the end up costs of production. The simplest process is the batch operation which yields the lowest cell densities. All the nutrients are provided at the initial stage and the cells grow until the nutrients run out. By continuing the growth phase and keeping the optimal conditions by introducing more concentrated nutrient solutions during the run, larger cell densities than batch are achieved in fed-batch operation. The pharmaceutical industry can be used to achieve yields of approximately 20 million cells/mm in fed-batch [45]. Medium is added and removed simultaneously at steady state in continuous (perfusion) mode, allowing extremely high cell densities and the longest culture times. Parameters elevation such as cell densities, doubling times, and bioreactor efficiency have shown significant cost reductions. A optimized continuous system minimised costs by USD 437,000 to USD 1.95/kg [7].

### 1.4.7. Challenges faced during scaling-up

To ensure cell viability, product safety, and batch to batch reproducibility, several important process parameters need to be tightly controlled during the manufacturing process, irrespective of the type of bioreactor. They include, temperature uniformity within the entire volume of the vessel; dissolved oxygen concentration, acidification due to lactate buildup quickly leads to cell death, shear stress which must be kept below cell damage thresholds of about 0.1-1 Pa in mammalian cells, and prevention of microbial contamination which is the main cause of batch loss in open or semi-open culture systems [8, 41].

All these properties change non-linearly as they are scaled up to commercial production out of the lab bench. With increase in vessel volume, the rate of oxygen transfer (OTR) decreases and surface aeration systems or sparging are necessary, posing further dangers of shear stress. Nutrient and metabolic wastes are producted in the vessel as mixing time increases. Even though recent studies have reported effective bioreactor expansions of above 50 L and a 90% cost savings in culture media, there is limited industrial translation [46].

## 1.5. Types of cells used to produce Cultivated Meat

The type of cells used is pivotal in any dressed meat system, impacting the design of the downstream bioprocess, the composition of the culture media, the necessity for pulpits, and the sensitive and nutritive characteristics of the dressed meat. The named original cell type should be largely proliferative and therefore suitable for large- scale product, able of isolation into the different cell types set up in the mature meat [46]. The types of cells that are presently under disquisition for dressed meat product are muscle satellite cells (MuSCs), convinced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) and fibro- adipogenic ancestor cells (FAPs), myoblasts and adipocytes or pre-adipocytes.

### 1.5.1. Satellite muscle cells

Muscle satellite cells are the resident adult muscle stem cells that lie sandwiched between the sarcolemma and basal lamina of each muscle fibre. The satellite cells or myogenic stem cells or myogenic progenitor cells can self-renew, regenerate, and hypertrophy between the sarcolemma and the basal lamina of skeletal muscle fibres. After muscle damage, regulatory myogenic factors are induced by the environment that stimulates satellite cell proliferation, differentiation and fusion to form new multinucleated muscle cells [47].

### 1.5.2. iPSCs

The most common transcription factors found to be expressed in somatic cell reprogramming include OCT4, SOX2, KLF4 and c-MYC (Yamanaka factors). These factors have to be used to transform a terminally differentiated somatic cell, typically from a skin biopsy or a biopsy of connective tissue, into an induced pluripotent stem cell (iPSC). An important reason cultured meat has been developed is its ability to derive cells with the characteristics of nearly any cell type, including pluripotent cells like induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), which can grow into almost anything and can be propagated indefinitely [46].

The iPSCs' can reproduce in-vitro, makes them plastic and suitable for applied research. Pluripotent stem cells (ESCs and iPSCs) allow generation of larger numbers of myogenic progenitor cells without tissue sampling repeats [47].

### 1.5.3. Mesenchymal stem cells

Multipotent adult stromal cells, also called mesenchymal stem cells (MSCs), have been discovered in skeletal muscle, adipose tissue, bone marrow and umbilical cord. The sources of cells for cultured meat are mesenchymal stem cells from tissues, self-renewal muscle progenitor stem cells in muscle tissues (most common), and induced pluripotent stem cells which are terminally differentiated cells that are transformed [48].

### 1.5.4. Fibro-adipogenic progenitor cells

A unique type of multipotent cells are called fibro-adipogenic progenitor cells (FAPs). They have the ability to transform into adipocytes and fibroblasts. These are the primary contributors to intramuscular fat, which is very important for the flavor, juiciness and eating quality of premium beef products. The cells derived from muscle-derived fibro-adipogenic progenitor cells are a good source for cultured bovine AT (adipose tissue). Fat tissue is necessary to precisely imitate conventional meat and is crucial to the flavor and texture of meat [49].

## 1.6. Types of culture media

Culture medium: nutritional medium that provides all molecular needs for cell survival, proliferation, and differentiation: energy sources, amino acids, vitamins, mineral salts and bioactive signalling molecules. Formulating the media is a commercially critical challenge in cultured meat production, accounting for at least 50% of the operating costs in cultured meat production [50].

These are added to standard basal media (most commonly DMEM or DMEM/F12) to provide key growth factors including FGF2, EGF, IGF1, NRG1, TGFβ1 and PDGFB, and serum proteins (albumin, insulin and transferrin) [51].

The traditional cell culture supplement, fetal bovine serum (FBS), is, as it happens, not really suitable for cultured meat purposes at all, and is normally included at 5–20% (v/v). The major cultivated meat production costs 90 percent that attribute to growth media which is why there is a need for low-cost FBS alternatives, moreover the various issues surrounding FBS lot-to-lot variability and contamination risks in food-grade manufacturing. The technology produces live fetuses by a cardiac puncture, directly opposing the ethical base of the technology [52].

Autocrine engineering is another important step, whereby cells are genetically engineered to manufacture their own growth factors. A media component that has proven to be very expensive, namely exogenous FGF2, may be avoided by engineering bovine muscle stem cells to express growth promoting signalling cascades that are usually activated in the presence of such exogenous growth factors [53].

An creative strategic use of microalgae as a source of nutrients derived from *Chlorella vulgaris* together with growth factors from conditioned medium was able to encourage proliferation of primary bovine myoblasts proposed a more sustainable alternative to nutrients derived from grain [54].

In addition to growth factors, fully animal-free production requires the elimination of the coating proteins of the extracellular matrix (fibronectin, laminin — animal derived; recombinant and synthetic RGD-peptide alternatives are under development), antibiotics (which need to be eliminated in closed sterile bioreactor systems) and cryoprotectants for cell banking [8].

The strategies for reducing the cost of media are reducing the contribution of growth factors, use of inexpensive plant-based raw materials for basal media and optimization of media recycling by perfusion systems [50].

The landmark study illustrated that the cost of cultured chicken could be high as USD 6.20/pound by producing cultured chicken meat in a perfusion bioreactor with an animal-component-free medium of USD 0.69/litre, which comes within the price range of premium organic chicken [44].

## 1.7. Scaffolds for Cultivated Meat

### 1.7.1. Why scaffolds are essential

Structured cultured meat production scaffolding requires knowing the hierarchical structure of native skeletal muscle tissue that any cultured meat substitute needs to mimic. Skeletal muscle is one of the complex tissues in animals, individual contractile muscle cells (myofibres) 50-100 µm in diameter and up to several centimetres in length and are grouped into fascicles, they are surrounded by layers of connective tissue known as extracellular matrix (ECM). The extracellular matrix keeps the tissue elastic, promoting cell adhesion, serve as a 3D space, and aslo regulates biological processes [55].

Scaffolding is depended upon the anchorage dependence of myogenic cells. The major cells of interest for cultured meat production such as primary satellite cells (myoblasts) must be physically attached to a solid material to survive, spread and divide. Cells are very dependent on environment and scaffolding plays a crucial role in establishing tissue geometry and cell distribution [56].

The other engineering factor is the diffusion limitation of the oxygen, and nutrient transfer in the dense biological tissues. Dissolving $O_2$ in the aqueous media and diffuses passively but has a maximum effective diffusion distance through cell dense tissue of 100 – 200 µm from the nearest nutrient source. Any construct thicker and denser than this distance will have hypoxic cells that start to die, creating a necrotic core in a construct without vascular or perfusion supply [8].

The scaffold plays an active biological role apart from attachment and nourishment that is in guiding cell behaviour. It is done by integrating and responding to mechanical features in the physical environment. A very significant elements dictating cell destiny is the scaffold’s stiffness, which is generally examined as Young's modulus (E), which is feasible, matches equivalent to original muscle tissue, which is approximately 8-17 kPa [55]. Commitment of myogenic cells are negatively impacted by very soft substrates, whereas differentiation of osteogenic cells is positively impacted by extremely hard substrates. The creation of parallelly organized myotubes, which give whole-cut meat having a distinctive fibrous appearance It is facilitated by topographical guidance cues from anisotropic scaffold structures (aligned fibres), which direct cells to align a common axis. The highly organized architecture of skeletal muscle (anisotropic) is mimicked by aligned fibres, which benefits cell orientation [57].

Edibility is an important parameter that makes cultured meat scaffolds a standalone from all previous tissue engineering techniques. Scaffolds are now only used as short-term structural supports in medical tissue engineering and later they are eventually taken out, replaced by the patient's own extracellular matrix, or left in place inside the body where they deteriorate over a time period without injury. In contrast, scaffolds meant for cultured meat must be eaten as part of the food product. Current synthetic scaffolds consist of the risk of producing hazardous byproducts, and while animal-derived materials such as collagen, and gelatin are considered safe for people to eat, they are frequently expensive and non-renewable. This has led to a shift towards the renewable plant-based materials that offer multiple benefits that are biocompatible, sustainable, and the ability to resemble the structure of traditional meat [56].

### 1.7.2. Plant polysaccharides

Biomaterials derived from plants, such as proteins and polysaccharides, offer many technological and economic benefits: nutritional value, biocompatibility and consumer acceptance, low cost. Cellulose is believed to be the most abundant polymer in nature, apart from being a major component of plant cell walls. Cultured meat scaffold is considered as the superior mechanical strength, chemical stability, moreover, biodegradability of cellulose make them a valuable option. Cellulose, starch and glucomannan are polysaccharide materials which are biocompatible, biodegradable, and inexpensive. These qualities make them suitable to be used in cultured meat scaffolds [9].

Cellulose is highly hydrophilic and has no natural cell binding motifs. It is therefore RGD peptide-surface functionalisation is required or coat with adhesion proteins to facilitate myoblast adhesion [10].

Alginate is a biomaterial extracted from the brown seaweed cell walls. It is a widely studied biomaterials in the field of food biotechnology, and tissue engineering. It can form stable hydrogels under mild and food-safe conditions by ionic cross-linking using divalent cations (Ca2+, Ba2+ and Sr2+). The concentration of crosslinking ions can be used to control the gelation process. Alginate is unsuitable as a scaffold material since it cannot provide cell attachment sites due to its ability to interact with divalent cations to produce a net structure. For this, various studies have focused on incorporating alginate with other biomaterials to reduce this effect. For instance, the alginate-cellulose hydrogel formed with the medulla of Undaria pinnatifida presents superior structural, physical and biological properties in comparison with alginate alone [9].

Another promising polysaccharide that is readily available from plants which can serve as a scaffold for cultured meat is starch. Enzymatic (such as microbial transglutaminase) and/or thermal (such as retrogradation) processing methods can increase the stability of starch and mimic the structure of ECM; chemical oxidation and/or blending with food-grade proteins or polysaccharides can also broaden the applicability of starch and develop scaffolds with more reproducible properties, mechanical properties, and ability to be fabricated using advanced techniques such as 3D bioprinting [58].

Chitosan is an alternative that derived from the deacetylation of chitin from crustacean shell and fungal cell wall. It is a unique cationic polysaccharide with intrinsic cell-adhesion properties and antimicrobial activity. Epitopes are positively charged at physiological pH which is beneficial for initial cell attachment by electrostatic interactions, without the need for RGD functionalisation. An extra added value is the antimicrobial activity of chitosan, especially against Gram Positive bacteria, for open culture systems where the contamination of the culture is a major operation problem [9].

### 1.7.3. Plant proteins

Soy protein isolate (SPI) is the most widely studied plant protein scaffold for cultured meat, because, apart from being highly proteinous (more than 90% on dry matter basis) it has excellent film-forming and gel-forming properties and has arginine-glycine-aspartate (RGD)-like sequences in its primary structure that, by themselves, give it partial cell adhesion capacity. Soy protein, pea protein, zein and glutenin are proteins that promote cell adhesion, proliferation and differentiation and are highly nutritious [9].

Bovine satellite cells cultured successfully on scaffolds that is made from a textured soy protein (TSP). It is basically a porous by-product of the soy protein in oil extraction which makes it one of the first plant-derived materials that was validated for use in cultured meat scaffolds [59].

With the plant-based food industry and lower allergenicity profile, pea protein isolate attracts significant research interest as an alternative to soy protein. For broader acceptance, pea proteins are relatively less allergenic, and are increasingly being considered for scaffold applications [60].

Zein is the major storage protein of maize, a hydrophobic prolamin soluble in aqueous ethanol, with outstanding film forming ability used widely in the food and pharmaceutical industries. Alginate hydrogel cell culture that is functionalized with RGD successfully integrated short-stranded fibres made from 28% zein solutions utilizing electrospinning and ultrasonication techniques. Another study used wet-spinning to create zein-alginate composite fibres with aligned architecture, which aligned the C2C12 myoblasts along the fibre axis. This parameter is crucial for the development of the closely aligned myotube bundles that give whole cut meat its texture and bite[9, 61].

The viscoelastic extensible network is one of the key components of cereal food technology, which is provided by wheat gluten. It is also available on a large scale as an agricultural commodity by-product, extremely low cost and exhibits good film-forming and surface properties, making it an interesting material for the production of scaffolds at a large scale. But wheat gluten is low in the essential amino acid lysine and is required by EU and US food labelling standards to be labeled as an allergen which is a practical limitation on the use of wheat gluten in final consumer product [9].

### 1.7.4. Decellularised scaffolds

Leaves containing the natural cellulose-pectin extracellular matrix (ECM) scaffold intact, including the native vascular network is an innovative method of making scaffolds for cultured meat products that directly addresses the diffusion limitation by using a biologically pre-engineered solution. To be particular, decellularised spinach leaves were identified to be cost-effective, sustainable, as well as ecological, and found to be similar to gelatin-coated glass on which cultured meat was earlier developed as an edible scaffold [62].

The spinach leaf scaffold is the most researched and popular type of decullarised scaffold. In a study the bovine satellite cells were seeded on the surface of decellularised spinach leaves, and cultured for 14 days, where myogenic cells were seen as viable, differentiated and aligned on the cellulose matrix, directly benefiting from the retained leaf venation network to provide a pre-formed perfusable channel system that supports the delivery of nutrients to cells throughout the construct [63].

Apple hypanthium tissue has also been a potential candidate for being used as scaffold. It had been studied that the induced pluripotent stem cells (iPSCs) adhere and grow on decellularised apples when kept for a 14-day culture period. Likewise, C2C12 myoblasts also adhere and survives on top of the decellularised apples over the same duration [10].

### 1.7.5. Scaffolds made of mycelium and fungi

The vegetative body of fungi, called mycelium, is a highly promising edible scaffold biomaterial, as it is both edible and sustainable, but has naturally a three-dimensional, porous fibre architecture without any need for fabrication process. The filamentous fungi fermentation can be optimised for large-scale in fermentation vessels, moreover the morphological, biochemical characteristics can be tailor-made to get the desired product. Mycoprotein has all of the amino acids that are essential and a protein digestibility-corrected amino acid score of 0.996, making it a complete protein source, with similar bioavailability as dairy milk. The study studies multiple species of edible fungi to know whether thay are capable of being a carrier for cultured meat. At the end it was concluded that the mycelium carriers of *Aspergillus oryzae* produced the greatest proliferation of myoblast cells (C2C12) among all the other edible fungi tested. The mycelium carriers also enabled the proliferation , and differentiation of bovine satellite cells, Ehich demonstrates the potential of edible mycelium carrier technology for the development of cultured meat products [64].

A second report showed the *P. ferulae* scaffold could be used to create high adhesion of cells, and aligned differentiation, and proliferation of BMC into organized parallel myotubes, with a texture profile and post-cooking browning index similar to that of the conventional beef [65].

## 1.8. Technology used to produce plant-based scaffolds

### 1.8.1. Electrospinning

The electrospinning technique is basically a fibre formation process, involving a polymer solution subjected to a high voltage (10–30 kV) electric field. Where the solution is drawn through a small nozzle, subsequent thinning occures to form a fine and continuous stream. Then it solidifies as it goes through moisture content and hits a grounded collector into nanoscale to microscale fibres. A structural analogue of the fibrous ECM architecture of skeletal muscle can be manufactured by electrospinning with agro-industrial protein by-products, such as zein from corn processing, soy protein isolate, and pea protein from oilseed and pulse processing [66].

The fibre diameter, alignment and porosity may be varied by controlling the concentration of the solution, the applied voltage, the shape of the collector and the flow rate of the solution. Structure similar to that of native muscle fascicles, the aligned fibre collectors are parallel electrodes that rotate to create fibre mats (anisotropic in nature) that align the myoblasts along the fibre axis. In addition to zein, soy protein isolate, pea protein, cellulose acetate, and methylcellulose, other plant-based materials that have been electrospun for cultured meat are currently being explored. The use of green electrospinning (with aqueous or food-grade alcohol solvents) has been a growing research interest over the last few years, and nanofibre scaffolds made of edible polysaccharides that are safe and proteins are ideal for cultured meat production due to their biodegradability, biocompatibility, biological activity and safety for consumption [10].

A major drawback of electrospinning is the production of thick scaffolds that are also fully 3D (typical electrospun sheets are fibre mats of limited thickness which can only be used for whole cut products after additional processing) [60].

### 1.8.2. 3D bioprinting

Three-dimensional bioprinting uses materials known as "bioinks" that can be deposited in layers with or without cells into a defined pattern and geometry, at the millimetre to sub-millimetre level. Bioinks can be biopolymer, including soy protein, pectin, alginate, agarose, cellulose and gelatin, and are used for food applications, where they possess crosslinking mechanisms that enable the formation of stable hydrogels in the printed construction while keeping the desired fluid rheological properties of the bioink [10].

The most relevant modality for cultured meat is extrusion-based bioprinting, a technique that extrudes a bioink with shear-thinning characteristics through a small nozzle, using pneumatic or mechanical pressure, to deposit a pattern of material in a programmed manner that results in a 3D construct. Hydrogels for cell-laden bioprinting using agarose, gellan, and blends of agarose plus proteins (pea and soy) were developed to be stable and biocompatible, where the proteins served to modify the viscosity and swelling behaviour, which favor cell growth; no cytotoxic effects were observed in leachates collected from these polysaccharide-protein scaffolds when tested with C2C12 myoblasts [67] .

These 3D printed pea protein isolate-enriched alginate bioinks yielded well-defined geometries using an edible removable agar bath support, which was adequate to achieve recovery of bovine satellite cells with rates of ~80-90% viability after printing for prolonged periods, allowing attachment, differentiation and maturation [68].

The significant advantage of 3D bioprinting over other scaffold fabrication techniques is the ability to include multiple materials and cell types within a single construct, with spatial control—the co-printing of muscle, fat and connective tissue precursor cells in defined geometries to recreate the macroscopic architecture of a whole-cut steak. There is one key drawback still: throughput: Current extrusion bioprinting speeds are still too slow to meet the volumes needed at commercial scale [60].

### 1.8.3. Decullarisation

The process of decellularisation is taken from medical tissue engineering, which is used to create scaffolds for making cultured meat using plant tissue as the substrate. Preservation of architecture of the extracellular matrix but removal of cellular substance. Scaffolds are placed in deionized water or buffer and long-term stored submerged in the same or lyophilized. The typical decellularization method includes repeated hexane/phosphate buffer treatment, which removes the leaf cuticles, SDS solution for about 5 days and 0.1% Triton X-100/10% bleach solution for about 48 hours, yielding a white transparent background with well-defined veins [69].

Decellularised plant sources as scaffold biomaterials have some advantages due to the structural and functional characteristics that promote various cellular functions such as cell adhesion, proliferation and differentiation, these happen via their preserved vascular system. Furthermore, their edibility and low cost facilitate safer production and economical scaffolds for cultured meat [9].

The main benefits of decellularisation of plants are: the vascular network is well preserved and can be perfused with culture media; no special synthetic chemistry is required, except simple laboratory "standard" chemicals; and the raw materials are cheap commodity food crops. The cons include the residual detergent cytotoxicity, and thier washing procedures has to be carried carefully in a controlled and validated manner inorder. to remove all of the detergent from the glassware before cells are seeded [69].

### 1.8.4. Freeze-drying

Among the most used fabrication methods for porous scaffold production for cultured meat, the simplicity, scalability and versatility of biopolymer used for freeze drying make it a very common method. To create a porous structure that is interconnected and whose pore size depends on the morphology of the ice crystals during the phase of freezing. Subsequently the sample has to be frozen at temperatures ranging between -20 degrees Celsius and -80 degrees Celsius, and then vacuumed to sublime the ice crystals from the solid to the vapour phase without passing through the liquid phase. The ease of use and low cost of freezedrying scaffold manufacturing are advantages, however achieving the pore connectivity and uniformity needed in this process can be tough. These issues are corrected by regulated the freezing speed and polymer concentration [9].

The overall porosity and size distribution can be controlled by modifying the freezing rates. Smaller and more uniform pores achieved when freezing faster, larger and highly irregular pores formed when under slower freezing conditions). This creates a very porous scaffold that is dry, light, and stable for long-term storage, and rehydrated just before adding cells into the scaffold with culture media [60].

### 1.8.5. Solvent casting and hydrogel

Solvent casting is considered to be the least complex and most readily available method for producing scaffolds. The process is cariried out by dissolving biopolymer in a food-grade solvent (usually aqueous-ethanol for zein, or water at a specified pH for soy and pea proteins) and cast into a mould of desired shape prior to the solvent forming a gel like or dried condition. Food-grade crosslinking agents enhance the mechanical stability and decrease the solubility of the scaffolds in the aqueous culture medium, with transglutaminase and calcium chloride scaffolds demonstrating higher compression modulus, water resistance and cell viability than uncrosslinked scaffolds, and genipin-crosslinked scaffolds representing the natural crosslinker derived from Gardenia jasminoides fruit [9].

## 1.9. Legal Regulations Worldwide

Actually, though, the regulatory status of cultivated meat is extremely variable, from full commercial approval to an active review process to preemptive legislative ban, not just because of the difference in food safety governance architecture, but because the prospect of changing the definition of meat in law is such a politically, culturally and economically charged topic. Cultivated meat products are rapidly progressing in both quality and quantity with the following significant milestones recorded in their trajectory: In December 2020, Singapore became the first country to approve the first cultivated chicken; and in June 2023, 2 cultivated chicken products from the USA were approved, while in January 2024, 2 cultivated chicken products from the USA were approved and in Israel, a cultivated beef product produced by Aleph Farms was approved. In contrast, an increasing number of countries – most recently Italy, but also some US states – have passed legislation that bans it, leading to a patchwork of international laws and regulations that sometimes leave the same product legally approved for sale in one country but prohibited in the adjacent one. Despite this uneven regulatory response, 174 companies worldwide are currently developing cultivated meat, not through animal farming or fishing [70].

A French start-up Gourmey is the first applicant to submit an application for regulatory approval of a cultivated meat product in the EU, intending to launch its product on the market: a cultivated duck foie gras. EFSA will perform a risk assessment of the product, which is supposed to take at least 18 months [71].

Israel has become one of the biggest hotspots in the world for cultivated meat research and business, with a concentration of the most prominent companies such as Aleph Farms, SuperMeat, and the now National Food Future Meat Technologies and Wilk Technologies. On 17th January 2024, the Israeli Ministry of Health (MOH) gave the world's first regulatory approval for a cultivated beef product made by local company Aleph Farms, to be produced and marketed in Israel, based on particular labelling and marketing directions and completion of an inspection of its pilot production facility, under the Good Manufacturing Practices (GMP) standard. Regulated according to the novel foods standard from the Food Standards Australia New Zealand (FSANZ) (Standard 1.5.1). FSANZ has at least one formal application (from Vow for cultivated quail) and is actively assessing other applications. In December 2021, China's Ministry of Agriculture and Rural Affairs announced its plan to boost innovation in frontier food technologies, which include cultivated meat, in the 14th Five-Year Plan. In 2025, the global regulatory landscape for cultivated meat is defined by a small number of markets with regulatory frameworks in place, including Singapore (2020), the United States (2023, with state-level variation), Israel (2024) and Australia (2024), a much larger number of markets that are in early stages of developing frameworks and a minority of markets with pre-emptive restrictions. A favourable regulatory environment has been developing in some countries, while others have decided to prohibit the technology and the international regulatory environment is characterized by wide conflict between food innovation policy and agricultural protectionism, consumer protection and ethics policies of governance – tensions which are unlikely to be easily solved [70].

The trajectory of EU regulation — and particularly the outcome of EFSA's risk assessments of the Gourmey and Mosa Meat applications — will be among the most consequential regulatory developments of the next five years for the global cultivated meat industry [72].

### 1.10. Literature summary

Global protein demand is estimated to rise by 35-56% from 2010 to 2050, however conventional livestock production, which accounts for 14.5% of anthropogenic GHG emissions and about one third of global freshwater usage, is not sustainable to meet this demand [1, 2, 14]. Partial solutions exist with alternative proteins, but none can provide a sensory and structural complexity that is comparable to whole cut meat [30, 31]. The field's progress on all four pillars of meat production (cell sources, culture media, bioreactor systems, and scaffold technologies) has uniquely positioned cultivated meat to fill this void, which has been realized since the 2013 burger demonstration [5, 7, 32]. The technology has now crossed the threshold toward commercialization: regulatory approvals have been obtained from Singapore, the United States, Israel and Australia in the last five years (2020-2024) [70], but the lack of a way to develop the scaffolds is still a major unsolved challenge for structured meat.

Since scaffolds are inevitably consumed as part of the final product, synthetic polymer materials are ruled out and plant derived biomaterials are identified as the most promising scaffolding paradigm – polysaccharides and proteins, such as zein [8-10]. Electrospinning is one of the fabrication techniques that generates fibrous structures that are similar to the skeletal muscle ECM architecture and that promote the alignment and differentiation of myoblasts [11, 66]. Zein is a food-grade maize prolamin that has already been evaluated as GRAS and has been proven to possess a very good fibre forming capacity, and electrospinning of zein is therefore the target of the experimental work reported in this thesis [11, 12].

## 2. Materials and research methods

### 2.1. Materials

The chemicals and reagents used in this study are listed in Table 1 below indicating the supplier, grade, specific function in the electrospinning process, and scientific justification for their use. Given its documented food-grade compatibility, biocompatibility and biodegradability along with its wide range of film and fibre formation properties, zein, the main prolamin storage protein of Zea mays L., was chosen as the scaffold-forming biopolymer [11, 12]. All additives (PEO, NaCl, glycerol, glucose) were chosen to be food grade, which is another demand as all of the scaffold factors must be considered safe for human consumption in the finalised cultivated meat product.

**Table 2.1** List of materials, reagents, and their characteristics

| Material / Chemical | Supplier | Role in Electrospinning | Key Properties / Justification |
|---|---|---|---|
| **Zein (corn prolamin protein)** | Sigma- Aldrich | Biopolymer used in scaffold formation. | GRAS (FDA); edible; biocompatible; biodegradable; fibre-forming capability. |
| **Ethanol ( 96%)** | Sigma- Aldrich | Primary solvent for zein dissolution, and it regulates solution viscosity and surface tension. | Food-grade; evaporates during electrospinning; safe for cell culture applications. |
| **Distilled / deionised water** | In-house | Co-solvent used to adjust solvent polarity and dielectric constant; modulates fibre morphology | Required to tune ethanol : water ratio and determines fibre diameter outcome |
| **Poly(ethylene oxide) — PEO** | Sigma- Aldrich | Enhances spinability; increases solution viscosity to stabilise the Taylor cone | Non-toxic; edible; widely used in electrospinning of protein solutions |
| **Sodium chloride (NaCl)** | Eurochemicals | Modifies conductivity; increases the solution’s electrical conductivity to improve fibre uniformity. | Electrolyte additive; reduces fibre diameter variability at higher electric field strengths [11] |
| **Glycerol** | Centro-Chem sp.j | Used as plasticiser, reduces brittleness of zein fibres. | GRAS; food-grade; widely used as protein film plasticiser [12] |

| **Table 2.1 (continued)** | | | |
|---|---|---|---|
| **Glucose** | Sigma- Aldrich | Secondary plasticiser / humectant. | Cell-compatible; functions as nutrient source in cell culture media. |

## 2.2. Research methods

### 2.2.1. Solution preparation

The biopolymeric zein solutions were dissolved in the aqueous ethanol solvent systems in varying ethanol to water ratios such as 60/40, 70/30, 80/20 and 90/10 (v/v) for different phases of the experiments. The preparation procedure for the standard reference solution (25% w/v zein, 70:30 ethanol: water, all additives) is described below:

- For the preparation of the required ethanol : water binary solvent, the required proportion of ethanol (96%) and deionised water (e.g., 70 mL of 96% ethanol + 30 mL deionised water for a 70:30 ratio) is mixed by volumetric addition and the required mass of zein powder is added and gradually mixed.

- According to the experimental condition (see Phase 6 — Additive Effect) add glycerol (7% w/w) and/or glucose (10% w/w). Stir the solution well (approx.12 hours), using a magnetic stirrer to make it homogeneous.

- If the solution becomes solid before preparation, discard and re-prepare the solution.

- After the homogenous condition is achieved, dissolve 0.5% w/v NaCl in the prepared ethanol: water solvent and subsequently add PEO (0.3% w/v) and keep in the magnetic stirrer for half an hour. This has to be done half an hour before the electrospinning process.

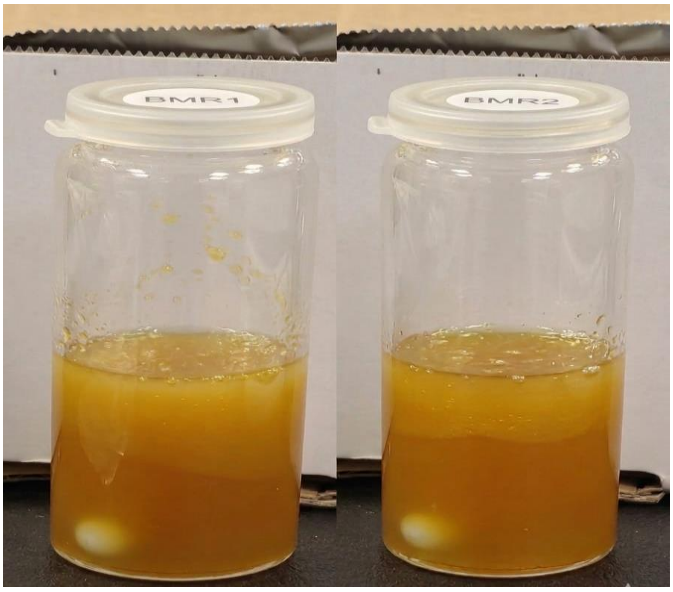


**Figure 2.1** Zein solution with all additives (PEO, NaCl, glycerol, glucose)

### 2.2.2. Electrospinning procedure

The following fixed environmental and instrumental conditions were used for all electrospinning experiments, unless otherwise indicated for the different experimental phases. Fixed environmental and instrumental conditions were used in all electrospinning experiments unless otherwise indicated for the different experimental phases. During the electrospinning process, continuously monitoring was done to observe jet stabilities, formation of Taylor cones and any irregularities in the process (irregularities include dripping, clogging, electro spraying) in each run.

### 2.2.3. Equipment and standard condition

- Equipment: Standard single needle electrospinning machine, high voltage power supply (10 to 20 kV DC), precision syringe pump, rotary drum aluminium foil collector and enclosed chamber.
- Needle type: 18 gauge needle (wall thickness: 0.216; dead volume: 14.011 µL/25.4 mm)
- Temperature was set at 24.5°C (controlled ambient, monitored throughout).
- Relative Humidity (RH): 35-40% (controlled ambient, monitored throughout). The evaporation of solvent may be slowed in high humidity, which leads to bead formation. Hence, this humidity level was chosen as a control and a reproducible environment.
- Collector: A rotary drum aluminium foil plate which is placed between the cliffs. All experiments used this type of collector, resulting in uniformly oriented non-woven fibre mats.
- The aluminium foil used as a collector was changed between runs, and cross-contamination was not allowed.

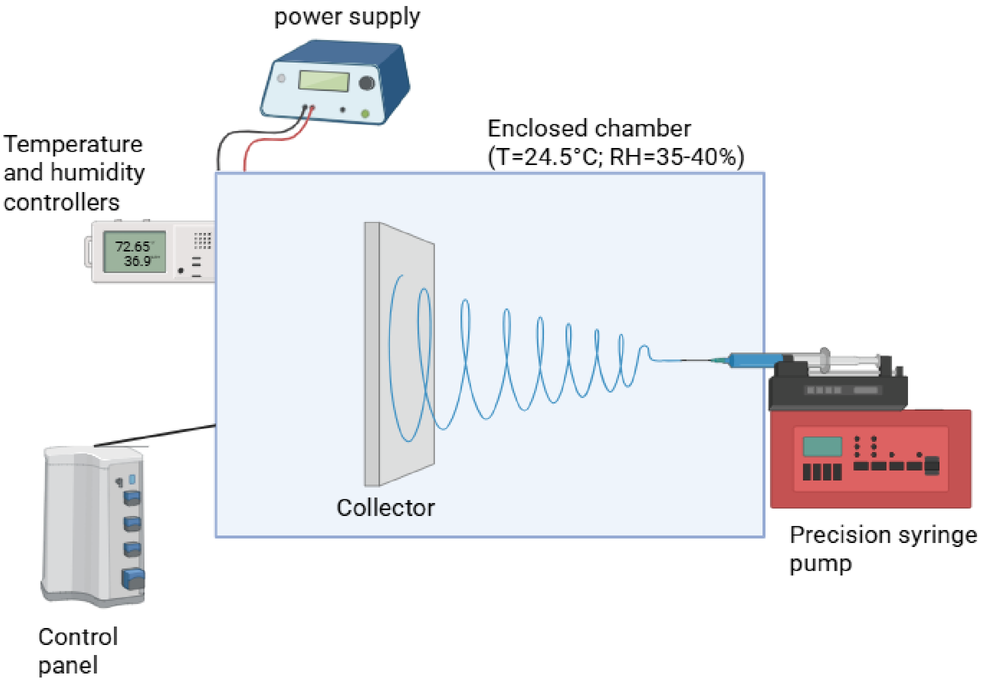


**Figure 2.2** Hardware scheme of electrspinning procedure

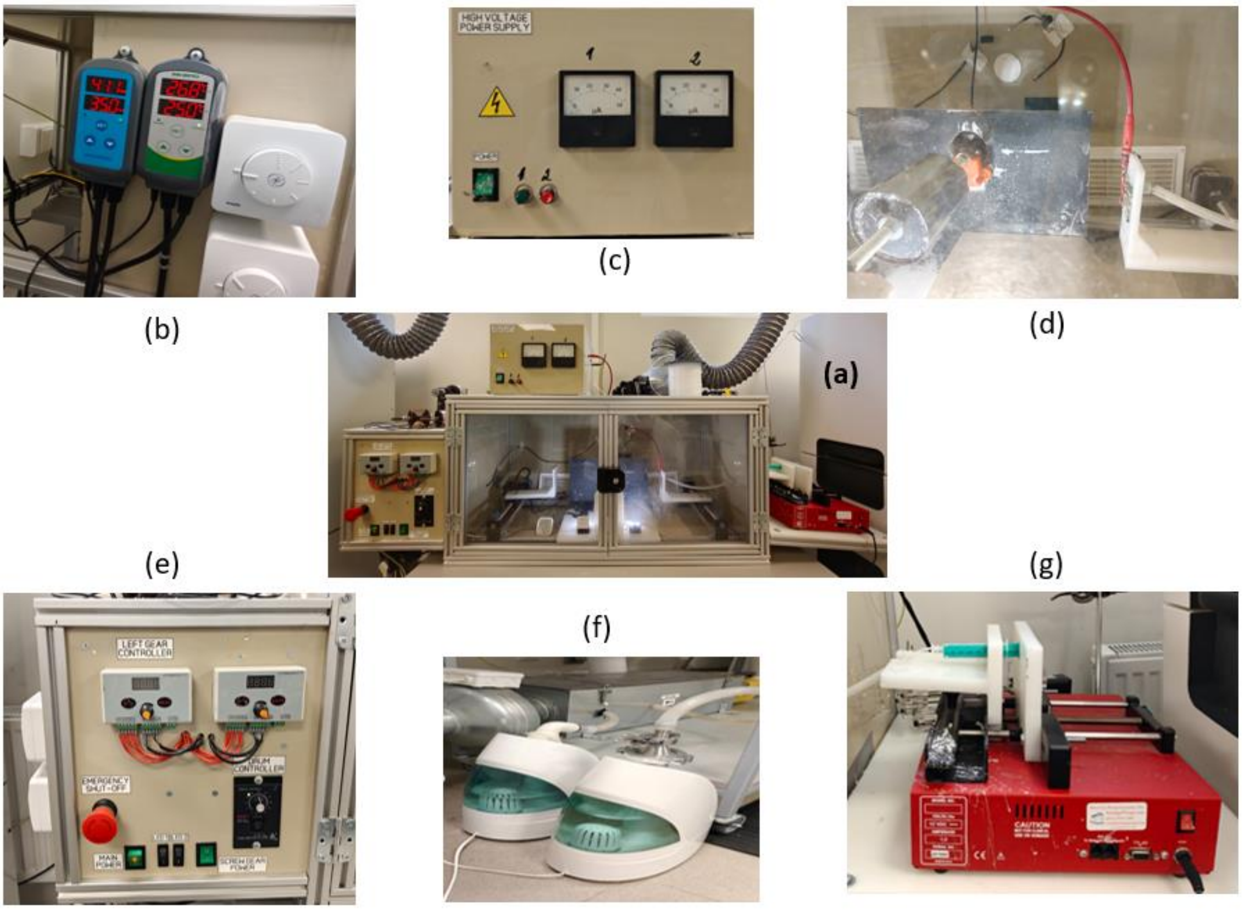


**Figure 2.3** Electrospinning apparatus used in this study

a) Electrospinning apparatus used in this study
b) Temperature (t) and humidity (H) controllers
c) Voltage power supply
d) Needle-to-collector zone
e) Drive and collector control panel with emergency shut-off.
f) Air Handlers / Humidity Conditioner for the environment control
g) Precision syringe pump

### 2.2.4. Electrospinning stepwise protocol

The zein solution was prepared and then filled into a syringe. During loading the solution into the syringe, the airbubbles formation was avoided. This is important because the air bubbles can lead to a flow disruption and destabilization of the Taylor cone in the electrospinning process. Then the syringe containing the solution was securely fitted in the syringe pump and the needle was inserted into the enclosed electrospinning chamber.

The precision pump was turned on once the syringe is been placed. The flow rate which was expected was set (mL/h). For the 18 gauge needle used in this work the dead volume was 14.011 µL per 25.4 mm.

The distance between the needle and collector was measured and checked using a calibrated ruler before each electrospinning process. This separation distance from the tip of the needle to the surface of the aluminum foil of rotating drum collector was measured. This measurement was made at the start of each process. Since a minor change in the position of the syringe mount or the collection setup may occur between each and every spinning process.

The high voltage power supply was turned on after the flow was stabilized and the distance was checked. The voltage level desired was not used immediately. On the contrary, it was raised step-wise by about 2 kV at a time until the desired voltage of the experimental stage was achieved. A stationary droplet of solution at the needle tip, under conditions of a large voltage pulse, will violently eject and not be drawn into a steady jet, thus the selection of this phasing technique was made

The needle tip was closely monitored for the ramp-up and during the run. The most simple sign of a successful electrospinning is the meniscus that is stable, symmetrical and conical at the needle tip as a long continuous stream of fibers is drawn to the collector. Stable runs were then categorized as seperate process, where the cone was found and the jet was constant and continuous. If the jet was intermittent, and droplets would intermittently form at the needle tip between pulsations, then this was considered to be a pulsing run. If there is no continuous jet at all, and the behaviour is dominated by the ejection of droplets, the run was classified as electro-spraying.

These stability classes were observed after every 5 minutes during each run. It was important to perform the measurements at regular intervals throughout the run to avoid the possibility that some runs might have started out at a steady rate, but later turned to dripping or pulsing mode due to fluctuations in back-pressure or the amount of solution in the syringe, particularly at the end of the run. Therefore, this recording which was observed was then used to gain a more clear and concise perspective of process behavior in each run rather than a single snapshot.

All fiber mats were collected over a time period of 15 minutes for each sample. Based on preliminary observation, the collection time of this sample was selected because short collection times yielded mats too thin and brittle for reliable SEM, FTIR and XRD characterization, and longer collection times (15 minutes) consistently resulted in mats of sufficient thickness and mechanical stability for characterization with SEM, FTIR and XRD, but not so thick that the lower layers were compressed or covered.

At the end of the 15 minute collection period, the voltage was turned off and then the syringe pump. This was done on purpose because, if the pump is turned off during the application of the voltage, there may be a residual electric force that pulls solution back out of the needle, causing a mess on the collector foil, and possibly contaminating the edge of the collected mat. When both of the aluminum foil and fiber mat were taken out, the aluminum foil with the fiber mat was softly removed from the rotating drum collector and laid down. Since freshly obtained zein fiber mats can be fairly delicate and prone to tearing or distortion if handled aggressively, the foil was handled carefully at this point.

Then the collected fibre mats were dried in the air until the next day at room temperature. This drying phase was to ensure that all water and ethanol that may have been in the fiber mat during collection was completely dried. For SEM, FTIR, and XRD characterization, the mats were dried overnight in air after air drying.

### 2.2.5. Experimental design

The experimental investigation was done in the sequential optimisation approach. Solvent ratio screening tests were conducted first to determine the optimal ethanol:water ratio for the electrospinning of zein fibres, for which the 70:30 solvent ratio proved to be most suitable, producing a stable bead-free fibre formation with a favourable pore architecture. To further validate the suitability of the solvents, flow-rate optimisation was also performed in 70:30 and 80:20 solvent systems, both of which achieved better scaffold morphology and pore characteristics for 70:30 solvent formulation. Based on this confirmation, the test for reproducibility was conducted with the 70:30 condition, and all subsequent optimisation steps, such as voltage, needle-to-collector distance, additive composition and zein concentration, were performed with the established 70:30 condition as the reference condition.

The individual variables were varied in each phase while the other parameters were fixed at the reference condition (25% w/v zein, 70:30 ethanol: water, 0.3% PEO, 0.5% NaCl, 7% glycerol, 10% glucose, 14 kV, 6 mL/h, 10 cm, 24.5°C, 35-40%RH). The symbol ▲ in Table 2 indicates the parameter (s) varied within each phase of the experiment.

Phase-wise experimental design with constant parameters (control) and variable parameters (▲) for each group of samples. All experiments were conducted at a temperature of 24.5 °C and humidity of 35-40% while the needles were of 18-gauge size.

**Table 2.2** Phase-wise experimental design

| **Phase / Sample ID** | **Zein % (w/v)** | **Ethanol:water ratio** | **PEO % (w/v)** | **NaCl % (w/v)** | **Glycerol % (w/w)** | **Glucose % (w/w)** | **Voltage (kV)** | **Flow rate (mL/h)** | **Distance (cm)** |
|---|---|---|---|---|---|---|---|---|---|
| **PHASE 1 — Screening Experiment: Solvent Ratio Selection [VARIABLE: Ethanol:water ratio \| CONSTANT: all other parameters]** | | | | | | | | | |
| BMS1 | 25 | 60/40 ▲ | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |
| BMS2 | 25 | 70/30 (Desired) | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |
| BMS3 | 25 | 80/20 ▲ | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |
| BMS4 | 25 | 90/10 ▲ | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |

**Table 2.2 (continued)**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **PHASE 2 — Flow Rate Influence [VARIABLE: flow rate (3–6 mL/h) \| CONSTANT: solvent 70/30, all other parameters]** | | | | | | | | | |
| BMF1 (70/30) | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 3 ▲ | 10 |
| BMF2 (70/30) | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 4 ▲ | 10 |
| BMF3 (70/30) | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 5 ▲ | 10 |
| BMF4 (70/30) | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 ▲ | 10 |
| BMF1 (80/20) | 25 | 80/20 | 0.3 | 0.5 | 7 | 10 | 14 | 3 ▲ | 10 |
| BMF2 (80/20) | 25 | 80/20 | 0.3 | 0.5 | 7 | 10 | 14 | 4 ▲ | 10 |
| BMF3 (80/20) | 25 | 80/20 | 0.3 | 0.5 | 7 | 10 | 14 | 5 ▲ | 10 |
| BMF4 (80/20) | 25 | 80/20 | 0.3 | 0.5 | 7 | 10 | 14 | 6 ▲ | 10 |
| **PHASE 3 — Voltage Influence [VARIABLE: voltage (10–18 kV) \| CONSTANT: 70/30, flow 6 mL/h, distance 10 cm]** | | | | | | | | | |
| BMV1 | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 10 ▲ | 6 | 10 |
| BMV2 | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 ▲ | 6 | 10 |
| BMV3 | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 16 ▲ | 6 | 10 |
| BMV4 | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 18 ▲ | 6 | 10 |
| **PHASE 4 — Needle-to-Collector Distance [VARIABLE: distance (10–25 cm) \| CONSTANT: 70/30, 14 kV, flow 6 mL/h]** | | | | | | | | | |

**Table 2.2 (continued)**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| BMD1 | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 ▲ |
| BMD2 | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 15 ▲ |
| BMD3 | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 20 ▲ |
| BMD4 | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 25 ▲ |
| **PHASE 5 — Additive Effect [VARIABLE: presence/absence of glycerol and glucose \| CONSTANT: 70/30, 14 kV, 6 mL/h, 10 cm]** | | | | | | | | | |
| BMA1 (no additives) | 25 | 70/30 | 0.3 | 0.5 | 0 ▲ | 0 ▲ | 14 | 6 | 10 |
| BMA2 (glycerol only) | 25 | 70/30 | 0.3 | 0.5 | 7 ▲ | 0 ▲ | 14 | 6 | 10 |
| BMA3 (glucose only) | 25 | 70/30 | 0.3 | 0.5 | 0 ▲ | 10 ▲ | 14 | 6 | 10 |
| BMA4 (glycerol+ glucose) | 25 | 70/30 | 0.3 | 0.5 | 7 ▲ | 10 ▲ | 14 | 6 | 10 |
| **PHASE 6 — Optimization: Zein Concentration [VARIABLE: zein % (15–25%) \| CONSTANT: 70/30, 14 kV, 6 mL/h, 10 cm]** | | | | | | | | | |
| BMC1 | 15 ▲ | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |
| BMC2 | 17.5 ▲ | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |
| BMC3 | 20 ▲ | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |
| BMC4 | 22.5 ▲ | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |
| BMC5 | 25 ▲ | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |

**Table 2.2 (continued)**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **PHASE 7 — Reproducibility Verification [FIXED: BMS2 optimum conditions (70/30) \| n=3 repeats]** | | | | | | | | | |
| BMR1 (rep 1) | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |
| BMR2 (rep 2) | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |
| BMR3 (rep 3) | 25 | 70/30 | 0.3 | 0.5 | 7 | 10 | 14 | 6 | 10 |

- **Phase 1 — Solvent Ratio Screening (BMS group)**

The first phase was merely a screening phase. The concentration of ethanol:water in the four samples was the only factor that was changed. The four ratios of ethanol to water was tested: 60/40, 70/30, 80/20, and 90/10. The concentration of zein, quantities of additives, voltage, flow rate and distance were all kept at the reference condition. The purpose was straightforward: find out which the solvent ratio provided useful fibres before trying any further optimisation.

BMS1 (60/40), BMS2 (70/30), BMS3 (80/20), and BMS4 (90/10), these are the four phase samples of ratio used in this phase. These four particular ethanol:water ratios were chosen because the ethanol/water ratio can have a profound effect on solubility, viscosity and surface tension simultaneously in zein solutions. Too little ethanol and zein and they will not dissolve properly, while too much and the solvent will quickly evaporate and the jet will not form properly. This phase resulted in the 70/30 condition which was used as the standard for each of the subsequent phases.

- **Phase 2 — Flow Rate Influence (BMF group)**

The next variable that has to be examined was flow rate after the solvent ratio was verified. This phase was tried under two solvent conditions (70/30 and 80/20) so that the two most promising ratios obtained in Phase 1 could be compared. Four flow rates were used here for each of the solvent condition: 3mL/h, 4mL/h, 5mL/h, and 6mL/h.

This showed an outcome of a total of eight samples: BMF1 under 70/30, BMF2 under 70/30, BMF3 under 70/30, BMF4 under 70/30, BMF1 under 80/20, BMF2 under 80/20, BMF3 under 80/20, and BMF4 under 80/20.

All other parameters were typically adjusted here to the reference value. For each solvent group, the flow rate of the syringe pump was the only variable; zein concentration was 25% w/v, the entire additive set was present, the voltage was 14 kV and the needle-to-collector distance was 10 cm. The amount of solution fed to the Taylor cone per unit of time is determined by the flow rate. At very low flow rates, the jet can become unstable and the cone can become starving.

At high flow rates there is excess solution at the needle tip, causing the cone to overload and leak. To cover both sides of this balance, a range of 3–6 mL/h was chosen.

- **Phase 3 — Voltage Influence (BMV group)**

In the third phase, the applied voltage was the next. The distance was established at 10 cm, the solvent was fixed at (70/30) and the flow rate on 6 mL/h. Samples BMV1, BMV2, BMV3 and BMV4 were acquired by testing four different voltage levels: 10kV, 14kV, 16kV, and 18kV. The voltage range 10-18kV was used for the electrospinning machine in this study, which is the practical operating window of the machine. Below 10 kV, the electrostatic force is usually not strong enough to break the surface tension of the zein solution to form a stable jet.

There is a problem of jet instability and uncontrollable whipping, above 18 kV. To assess both ends of the range, the four voltage points, 10, 14, 16 and 18 kV, were spread across the range, with the two points in the higher end of the range where instability effects were more noticeable.

- **Phase 4 — Needle-to-Collector Distance (BMD group)**

Phase 4 is basically looking at the significant distance between the needle and collector. The flow rate was 6 mL/h, voltage was 14 kV and solvent was 70/30. The three distances (10, 15 and 20 cm) were characterized by using SEM and the samples were named BMD1, BMD2 and BMD3 respectively. Additionally, a fourth condition at 25 cm (BMD4) was also run and was monitored for process stability but was not carried forward for SEM characterisation.

The strength of the electric field between the needle and the collector, and also the amount of time available for solvent evaporation during the jet's journey, are both simultaneously affected by distance. The longer the distance, the weaker the electric field, but also the longer the time for the solvent to evaporate before the fibre lands, and the shorter the distance, the stronger the electric field, but also the shorter the time for the solvent to evaporate before the fibre lands. A distance range of 10–25 cm was chosen to explore both extremes of this trade-off within the realistic (enclosed) geometry used for the electrospinning process.

- **Phase 5 — Additive Effect (BMA group)**

The function of the two plasticising ingredients, glucose and glycerol, in the formulation were examined in phase 5. Four combinations of additives were evaluated under the same fixed conditions: solvent 70/30, zein 25%, 14kV, 6 mL/h and 10cm distance. The amount of glycerol and glucose used was as follows: BMA1: no glycerol and no glucose (baseline without plasticisers); BMA2: glycerol at 7% w/w and no glucose; BMA3: no glycerol and glucose at 10% w/w; BMA4: glycerol at 7% w/w and glucose at 10% w/w; PEO and NaCl were kept at 0.3% w/v and 0.5% w/v respectively in all four samples. This phase design was not based on a concentration gradient of the two additives, but on a simple inclusion/exclusion logic as the main questions concerned whether glycerol and glucose were necessary separately, whether there was an interaction between them, whether the combination was more beneficial than either additive alone, or less.

- **Phase 6 — Zein Concentration Optimisation (BMC group)**

In phase 6, five different concentrations of zein (15%, 17.5%, 20%, 22.5%, 25% w/v) were studied which resulted in getting five different samples namely BMC1, BMC2, BMC3, BMC4, BMC5. The voltage was 14 kV, flow rate 6 mL/h, distance 10 cm, the proportion of the fixed solvent 70/30 and all additives in their usual proportions. The concentration range (15–25%) was selected to include the lower concentration at which zein solutions in aqueous ethanol showed sufficient polymer chain entanglement to create a continuous fibre and the upper limit of 25% was used as a reference point for all previous levels. Testing at lower concentrations was important as lower concentration would lead to lower material costs and lower viscosity of the solution (both of which would have practical implications for scaling up the process if it were ever to be implemented). The five concentration points were not evenly spaced; the first two, 15% and 17.5%, are 2.5% apart; the remaining points are 2.5% apart as well up to 25%. This provided a decent resolution range with not too many samples.

- **Phase 7 — Reproducibility Verification (BMR group)**

The last stage was not at all an optimising stage. The reproducibility testing was simple. Three electrospinning operations, BMR1, BMR2 and BMR3 were carried out using identical parameters: 25% zein, 70/30 solvent, all additives, 14 kV, 6 mL/h and 10 cm. All these conditions are a perfect fit to BMS2 ideal conditions in Phase 1. Three independent replicates were made to assess if the 70/30 reference condition gives consistent fibre shape from run to run or if there is significant variation between different batches of fibre prepared and spun on various dates. This is a basic, yet important, evaluation that should be made before determining conditions are ideal. If the procedure is not repeatable, then changes in conditions for previous stages cannot be related to the variable being changed.

## 3. Results and discussion

The results of all experimental runs are presented in Table 3, where the results for process stability, optical microscopy observations, fibre morphology interpretation from SEM images, quantitative fibre diameter, pore size measurement, and scientific explanation of the results for each experimental run are included. SEM imaging is the most important characterisation technique for fibre morphology as it gives direct, high resolution visual information on fibre shape, surface texture, fibre diameter distribution, inter-fibre spacing and pore architecture on a scale (between 100 nm and 1 mm) inaccessible to optical microscopy. For zein electrospinning from ethanol-water solvents, the expected morphology is ribbon-shaped fibres because the volatile solvent evaporates rapidly, leading to the outer surface of these fibres solidifying prior to the inner core [73, 74]. However, bead formation by contrast will signal too low a degree of polymer chain entanglement or perhaps too high a surface tension compared to viscoelastic forces of the jet [73].

Process stability, fibre morphology. Fibre diameter (Ø) and pore size values derived from SEM image analysis (ImageJ). Fibre diameter reported as mean (µm). Pore size reported as mean pore area equivalent diameter (µm).

**Table 3.1** Experimental outcomes for all electrospinning runs

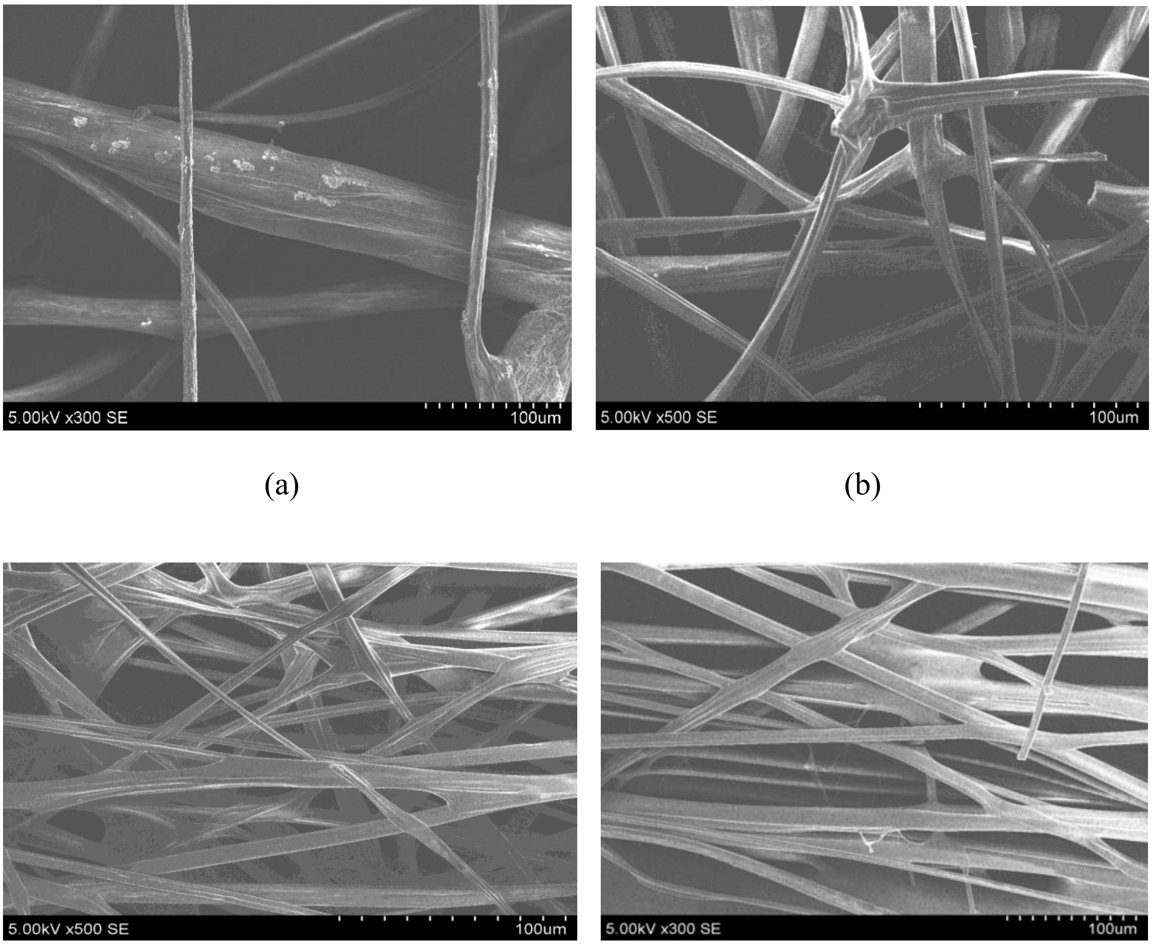
| **Sample ID** | **Ethanol:water / Parameter changed** | **Electrospinning stability** | **Fibre morphology (Optical)** | **Fibre form** | **Beads** | **Fibre Ø (µm)** | **Pore size (µm)** |
|---|---|---|---|---|---|---|---|
| BMS1 | 60/40 | Pulsing | Not uniform; beads present | Ribbon-like | Yes | 13.9 | 40 |
| BMS2 | 70/30 | Stable | Uniform; no beads | Ribbon-like | No | 10.1 | 64.2 |
| BMS3 | 80/20 | Stable | Uniform; merged fibres / cracks | Ribbon-like | No | 7.4 | 73.7 |
| BMS4 | 90/10 | Electro spraying | Not uniform; beads | Ribbon-like | Yes | 11.6 | 73.6 |
| BMR1 | 70/30 rep.1 | Stable | Uniform | Ribbon-like | No | 13.1 | 60.6 |
| BMR2 | 70/30 rep.2 | Stable | Uniform | Ribbon-like | No | 10.4 | 56.1 |
| BMR3 | 70/30 rep.3 | Stable | Uniform | Ribbon-like | No | 14.9 | 70.8 |
| BMF1 (70/30) | Flow 3 mL/h | Stable | Uniform | Fibres | No | 10.5 | 46.9 |
| BMF2 (70/30) | Flow 4 mL/h | Stable | Uniform | Fibres | No | 11 | 68.4 |

**Table 3.1 (continued)**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| BMF3 (70/30) | Flow 5 mL/h | Stable | Uniform | Fibres | No | 13.6 | 57.9 |
| BMF4 (70/30) | Flow 6 mL/h | Stable | Uniform | Fibres | No | 9.6 | 46.6 |
| BMF1 (80/20) | Flow 3 mL/h | Stable | Merged fibres/cracks | Merged fibres | No | 8.1 | 37.3 |
| BMF2 (80/20) | Flow 4 mL/h | Stable | Merged fibres/cracks | Merged fibres | No | 9.7 | 52.2 |
| BMF3 (80/20) | Flow 5 mL/h | Stable | Merged fibres/cracks | Merged fibres | No | 12.1 | 33 |
| BMF4 (80/20) | Flow 6 mL/h | Stable | Merged fibres/cracks | Merged fibres | No | 12.6 | 49.5 |
| BMV1 | 10 kV | Stable | Uniform | Fibres | No | 12.1 | 57.6 |
| BMV2 | 14 kV | Stable | Uniform | Fibres | No | 9.5 | 48.1 |
| BMV3 | 16 kV | Stable | Uniform | Fibres | No | 12.7 | 28.9 |
| BMV4 | 18 kV | Stable | Uniform | Fibres | No | 9.5 | 51.2 |
| BMD1 | 10 cm | Stable | Uniform | Fibres | No | 11.1 | 77.1 |
| BMD2 | 15 cm | Stable | Uniform | Fibres | No | 9.9 | 50 |
| BMD3 | 20 cm | Stable | Uniform | Fibres | No | 11.4 | 64.5 |
| BMA1 | No additives | Stable (late dripping) | Uniform | Fibres | No | 7.81 | 51.1 |
| BMA2 | Glycerol 7% | Stable | Uniform | Fibres | No | 10.7 | 56.2 |
| BMA3 | Glucose 10% | Stable | Uniform | Fibres | No | 11.7 | 44.2 |
| BMA4 | Glycerol + Glucose | Stable (late dripping) | Uniform | Fibres | No | 8.9 | 45 |
| BMC1 | 15% zein | Stable | Uniform | Fibres | No | 13.1 | 41.9 |
| BMC2 | 17.5% zein | Stable | Uniform | Fibres | No | 17.8 | 38.4 |
| BMC3 | 20% zein | Stable | Uniform | Fibres | No | 13.8 | 53.5 |
| BMC4 | 22.5% zein | Stable | Uniform | Fibres | No | 14.4 | 58.1 |
| BMC5 | 25% zein | Stable | Uniform | Fibres | No | 15.6 | 61.9 |

### 3.1. SEM interpretation

The morphology zein-fibrous scaffolds samples were evaluated using a scanning electron microscope (SEM - Carl Zeiss EVO MA10, Germany). The samples were cut into appropriate length, mounted on SEM stubs and examined at appropriate magnifications to consider the surface morphology. Digital photos were analysed using ImageJ software (N.I.H., USA) for this purpose. To ensure representative measurements along the length of the filament, each SEM image was divided into smaller segments, and calibrated based on the scale bar of the SEM. The diameter of the scaffold was calculated by measuring the average diameter at several places at right angles in each part. Other surface characteristics, so as the pore size.



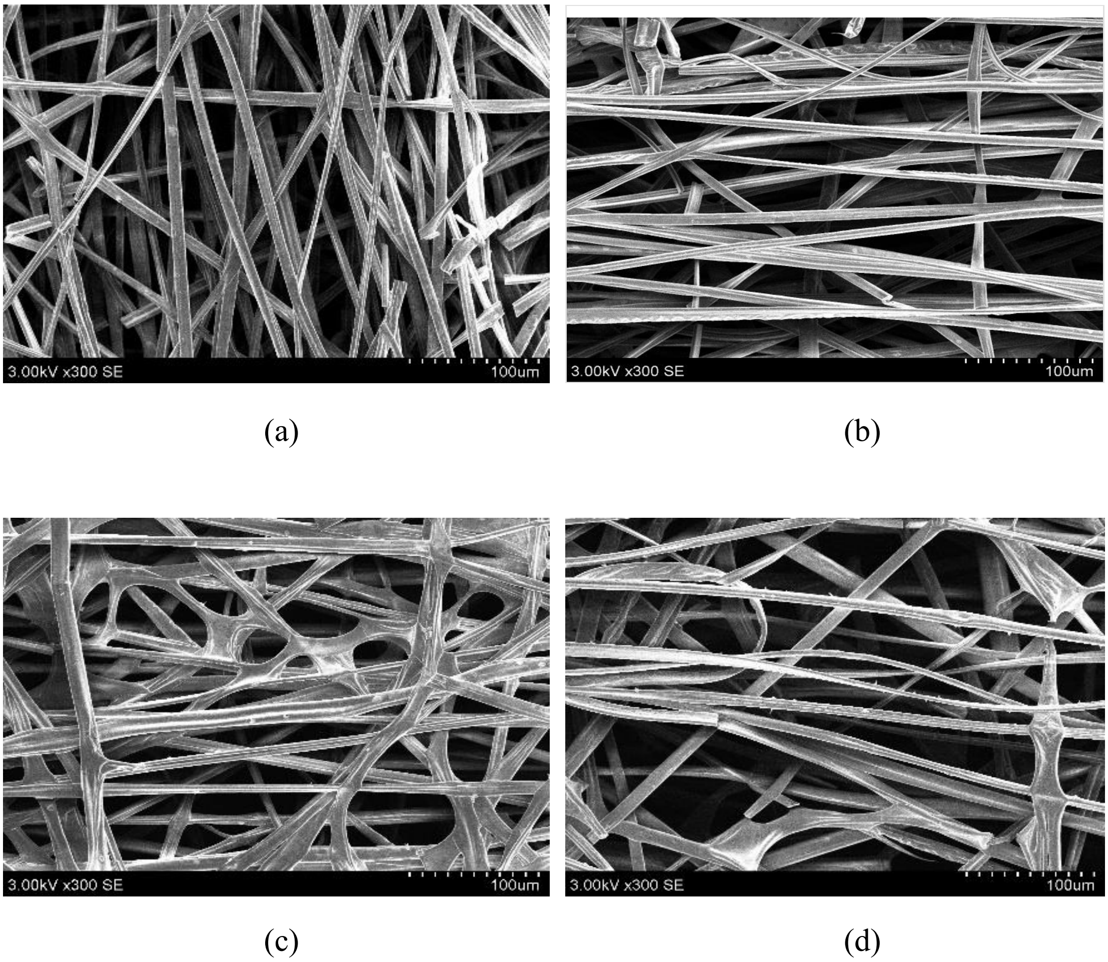
(a) (b)

(c) (d)

**Figure 3.1** SEM micrographs of the BMS fibres produced with different solvents ratios: a) BMS1 (60:40), (b) BMS2 (70:30), (c) BMS3 (80:20), and (d) BMS4 (90:10)

The ratio of the solvent was the most significant processing parameter on fibre morphology as compared to other processing parameters investigated. A change of ethanol:water ratio in the BMS series was clearly observed to influence fibre formation, pore structure and process stability. The higher ethanol content (BMS1, 60:40 ethanol:water) seemed to decrease zein solubility, zein solution viscosity and increase surface tension. This resulted in a less stable electrospinning jet, which created beads, irregular fibres and a relatively high fibre diameter of 13.9 µm. The pore size was also relatively small at 40 µm, which meant that the scaffolds were not very porous.

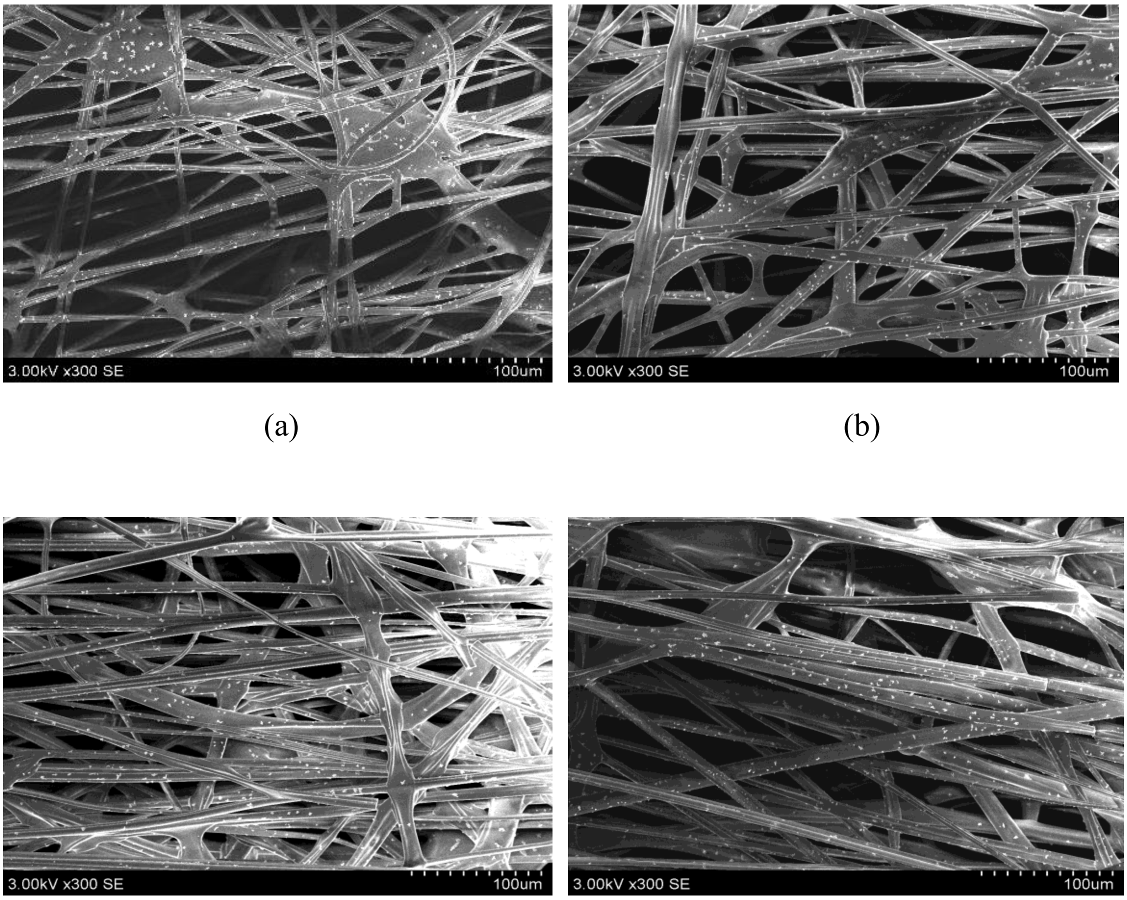
The optimum morphology was obtained for the BMS2 prepared in this study with a ratio of 70:30 ethanol:water because it displayed the most favourable morphology. The ribbon-like fibres obtained from this formulation had the lowest fibre diameter of 10.1 µm and the highest pore size of 64.2 µm with bead-free fibres. The stability of the Taylor cone formation and continuous stretching of the jet was achieved, which indicated that these features were suitable for obtaining a balance between zein solubility, viscosity, surface tension and solvent evaporation rate in the 70:30 ratio. Hence, BMS2 was determined to be the best solvent condition and it was therefore chosen as the reference formulation for further stages of the optimisation. With further increase of ethanol concentration to 80:20 in BMS3, it seemed that ethanol evaporation significantly influenced the fibre formation process, prior to final deposition on the collector. This caused some merging of fibres and surface cracking, but the numerical measurements of the pores were good, thereby decreasing the effective openness of the scaffold. When the ethanol content was the highest, 90:10 in BMS4, the evaporation rate was too high and fibre formation was not possible with the jet. On the other hand droplet-like structures and short fibre fragments were observed, suggesting that there is a possible transition from electrospinning to electrospraying. This shape was not conducive to scaffold construction.



(a) (b)

(c) (d)

**Figure 3.2** SEM micrographs of the BMF (70/30) fibres produced with varying flow rates: a) BMF1 (3 mL/h), (b) BMF2 (4 mL/h), (c) BMF3 (5 mL/h), and (d) BMF4 (6 mL/h)



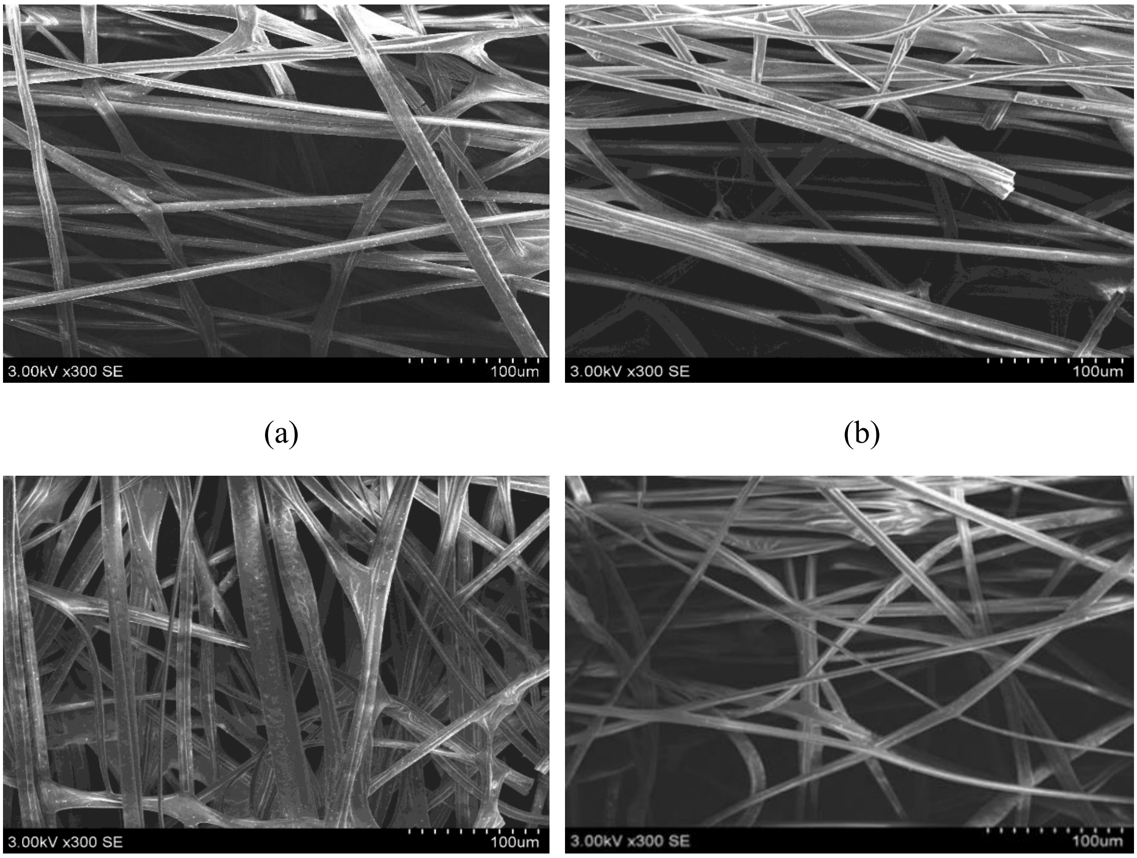
(a) (b)

(c) (d)

**Figure 3.3** SEM micrographs of the BMF (80/20) fibres produced with varying flow rates: a) BMF1 (3 mL/h), (b) BMF2 (4 mL/h), (c) BMF3 (5 mL/h), and (d) BMF4 (6 mL/h)

The optimal solvent ratio (70:30 ethanol:water) was determined and the BMF series was used to evaluate the effect of flow rate. The amount of polymer solution fed to the Taylor cone and hence the fibre thickness and fibre packing and pore formation was affected by the flow rate. BMF1 produced relatively fine fibres at low flow rate (3 mL/h), 10.5 µm. The lower polymer supply however, also led to closer fibre packing, which led to a lower pore size of 46.9 µm. BMF2 scaffold morphology was found to be the most favourable when the flow rate was increased to 4 mL/h in this series. The diameter of fibres remained uniform as 11.0 µm and the pore size was increased to 68.4 µm. This indicates that the amount of polymer delivered was appropriate, but not too high, to give the required electrostatic stretching to form stable fibres and a more porous network. This condition was then deemed the most appropriate flow rate to achieve an architecture of the scaffolds which would be suitable to infiltration by cells. The fibres are broader and the mat density was higher for the higher flow rate of 5 and 6 mL/h. This resulted in a reduction of porosity. This implies, if the flow rate is excessive, it can lead to overloading of the Taylor cone and the effectiveness of the scaffold will be shorter, so the jet stretching process will not be more efficient. However, with the 80:20 solvent system, it was not possible to compensate merging and cracking of fibres which possibily occurred as a result of the ethanol rapidly evaporating from the mixture, therefore flow rates had to be altered.

This shows the significance of the ratio of solvents in this instance, as it affected the morphology of the fibres, and the importance of flow rate as an optimisation parameter.



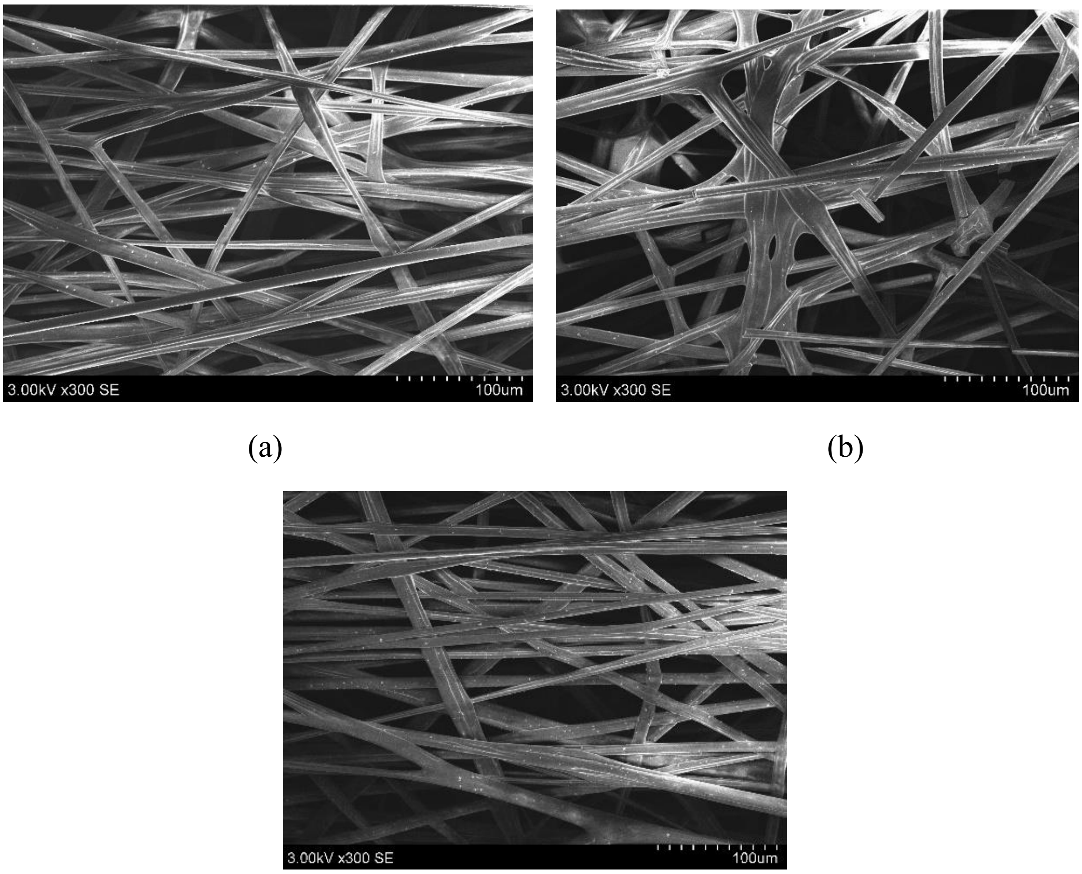
(a) (b)

(c) (d)

**Figure 3.4** SEM micrographs of the BMV fibres produced with varying voltage: a) BMV1 (10 kV), (b) BMV2 (14 kV), (c) BMV3 (16 kV), and (d) BMV4 (18 kV)

Application of voltage had an influence on fibre morphology through modification of the electrostatic force applied to the charged polymer jet. Overall, jet stretching is generally expected to be greater at higher voltages and fibre diameter is generally expected to be smaller at higher voltages. In the BMV series, however, the reaction was not entirely linear and it was found that too much electrical force also caused jet instability. Relatively wider fibres were obtained in BMV1 at 10 kV with a diameter of 12.1 μm and a pore size of 57.6 μm that is moderate. This means that the electric field was strong enough to form the fibres but not as strong as the high voltage electric field. BMV2 had thinner fibres (9.5 μm) and pore size (48.1 μm) at 14 kV. This condition served as a reference voltage since it gave stable and reproducible fibre formation.

In case of BMV3 (16 kV), the unexpectedly the increase in fibre diameter of 12.7 μm and the decrease in the pore size to 28.9 μm. This suggests that the electric field was stronger and would have led to instability and irregular jet deposition, which led to a denser fibre network. BMV4 again gave rise to thinner fibres (9.5 μm) and a better pore size (51.2 μm) at 18 kV. This could be due to enhanced whipping motion of the jet that increases spacing between fibres during deposition. The voltage response was overall non-monotonic and 14 kV was deemed the appropriate operating voltage for this zein-based electrospinning system.



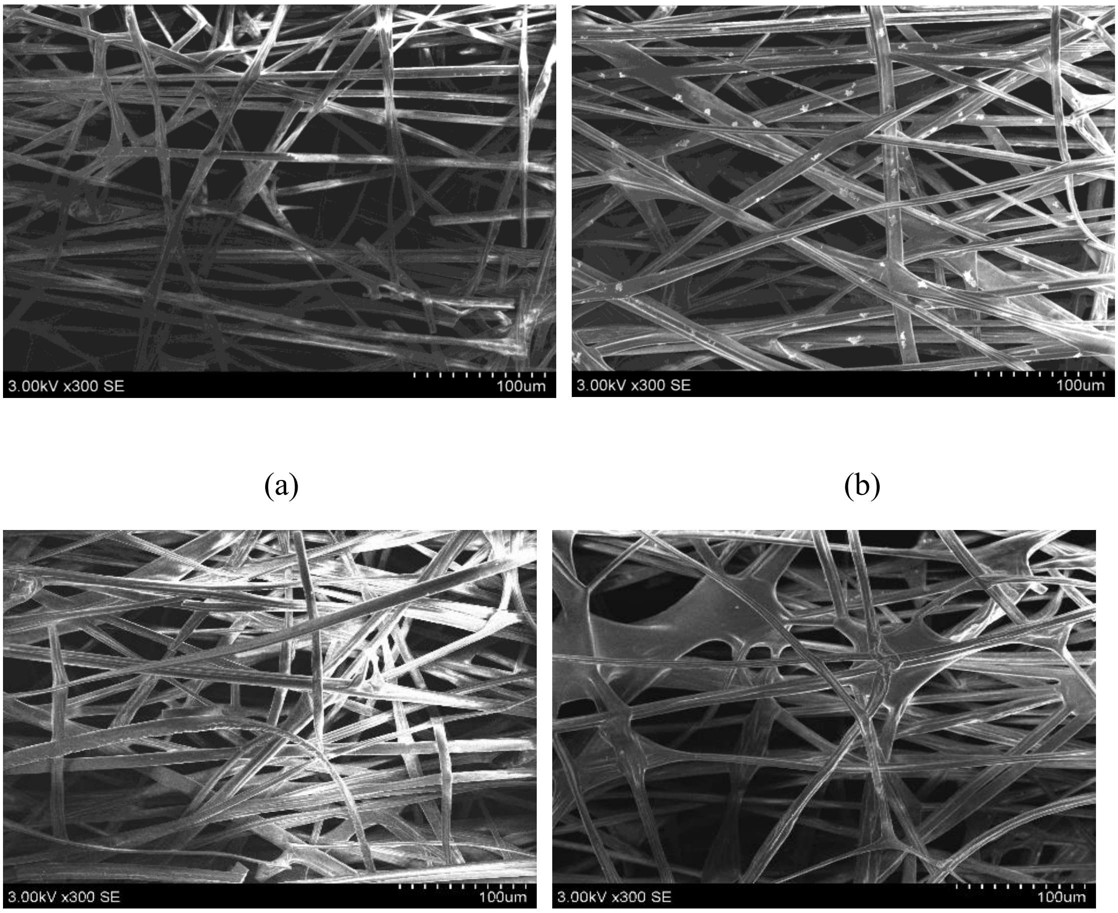
(a) (b)

(c)

**Figure 3.5** SEM micrographs of the BMD fibres produced with different needle to collector distance : a) BMD1 (10 cm), (b) BMD2 (15 cm), (c) BMD3 (20 cm)

The distance between the needle and the collector had an effect on fibre morphology as it controlled the flight time of the jet and the strength of the electric field. A shorter distance may keep an adequate electric field and a longer distance may allow for jet whipping and evaporation of the solvent. So the final fibre structure is related to this balance between the two effects.

In this series, the morphology was best on BMD1 at 10 cm. The fibres were well separated, ribbon-like and had a fibre diameter of 11.1 μm and the maximum pore size of 77.1 μm. This indicates that holding the distance of 10 cm was enough to allow solvent evaporation during the flight and also the electric field was strong enough to assure stable fibre stretching and deposition. In case of BMD2 reduction of the pore size to 50 μm was observed. This may be due to the weakening of the electric field strength, that have prevented the jet from stretching and subsequnetly allowed the deposition of denser fibres. BMD3 at 20 cm had recovered a little of the pore size of 64.5 μm which may be due to the longer flight path causing more whipping and hence more random fibre arrangement. The 25 cm condition, BMD4, was not characterized by SEM, but was found to be process stable. To conclude from the outcomes, 10cm was chosen as the optimum distance as it has the most open and clear fibre network.



(a) (b)

(c) (d)

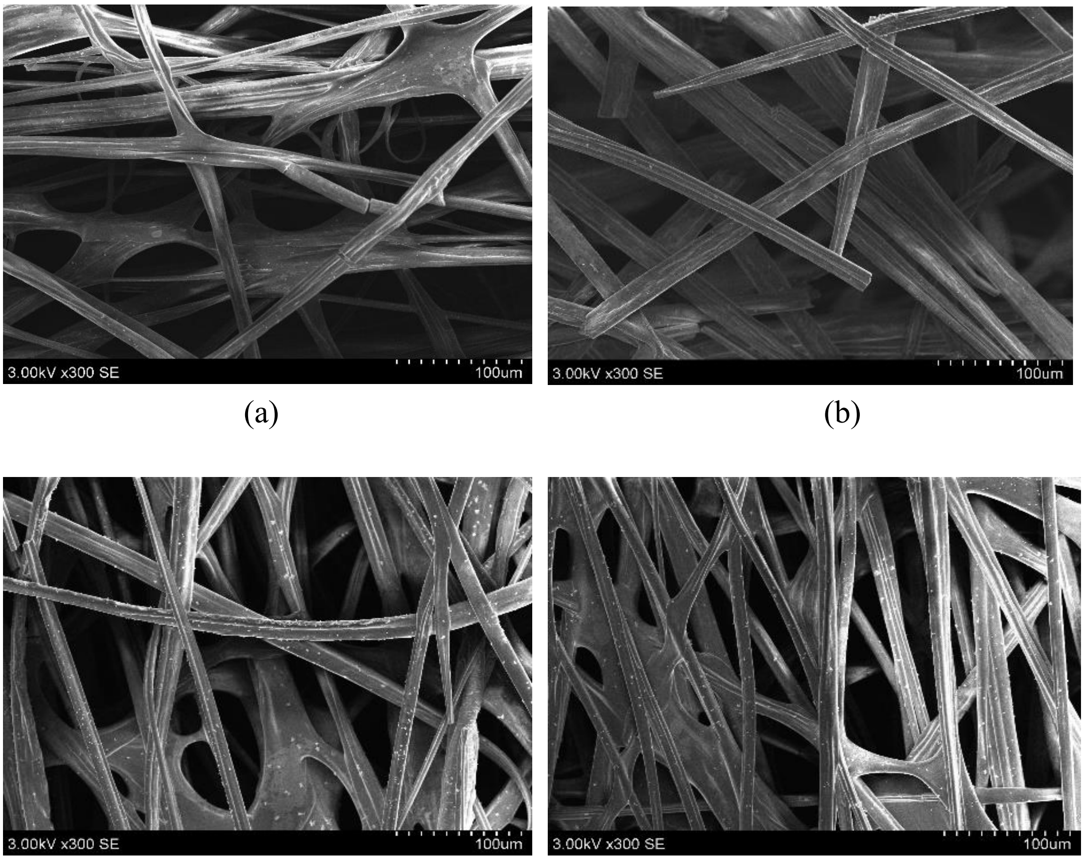
**Figure 3.6** SEM micrographs of the BMA fibres produced with varying additives: a) BMA1 (no additives), (b) BMA2 (only glycerol), (c) BMA3 (only glucose), and (d) BMA4 (glycerol+glucose)

Additive composition caused noticeable and significant modification of the fibre morphology and process stability. The presence and absence of glycerol and glucose had an impact on the diameter of fibres, pore size and stability of electrospinning in the BMA series.

The smallest fibre diameter in this series was obtained by BMA1 without any additives (7.81 µm). The process was, however, marginally stable with a late stage of dripping being seen during the electrospinning process. The pore size was also relatively low at 51.1 µm indicating that the fibre mat was relatively dense. This means that it was not possible to achieve the same process stability without plasticisers, but that thinner fibres can be made with them. The glycerol in BMA2 helped to enhance the electrospinning behaviour. Glycerol gave rise to an increased plasticity of the solutions and probably contributed to an increase in the mobility of the chains, which led to stable fibre formation during the process. The fibres were wider but the pore size was larger (10.7 µm vs. 56.2 µm). This indicates that the glycerol may have led to an increased open, stable fibre network which is desirable for the scaffold formation.

BMA3 yielded the lowest diameter of pores found in this study (44.2 µm). This may be due to the hygroscopic properties of glucose that may help to slow complete fibre drying prior to deposition. This means that fibres may be collected partially wet and may have been pressed against each other which decreases the space between the fibres. This is significant for scaffold application as cell infiltration might be hindered from scaffolds with smaller pore sizes.

The combined glycerol–glucose formulation (BMA4) led to a better balance of morphology (8.9 µm fibre diameter and 45.0 µm pore size). The dripping phenomenon was again observed at the end of the process, though, which suggests that process stability has not yet reached the desired level. The overall best additive effect was observed for glycerol as it not only provided better process stability, but also enabled a more open scaffold structure.



(a) (b)

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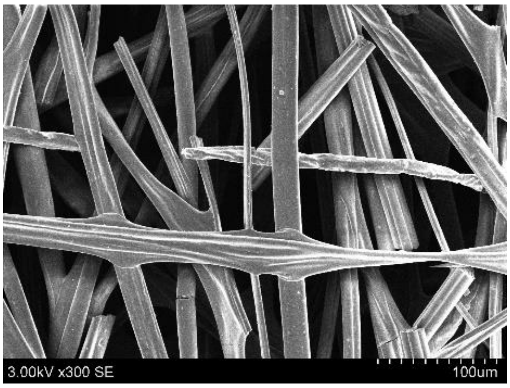


(e)

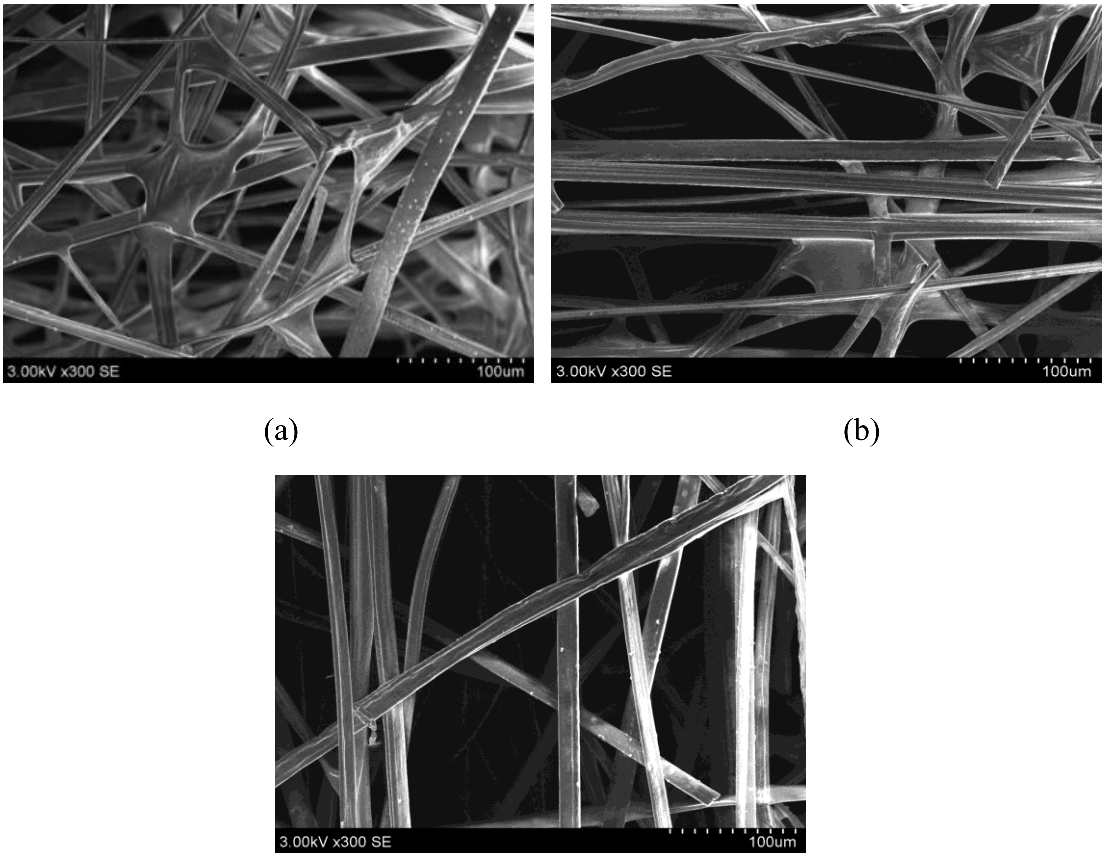
**Figure 3.7** SEM micrographs of the BMC fibres produced with varying zein concentrations: a) BMC1 (15% w/v), (b) BMC2 (17.5% w/v), (c) BMC3 (20% w/v), (d) BMC4 (22.5% w/v), (e) BMC5 (25% w/v)

The concentration of zein affected the fibre formation significantly, which consequently affected by the polymer chain entanglement and the solution viscosity. For BMC series, it was observed that the zein concentration level was varied from 15% to 25% which resulted in changes in fibre diameter and pore architecture.

Fibres from BMC1 were fine but less uniform due to its low entanglement (15%). The fibre diameter was 13.1 μm and the pore size was relatively small at 41.9 μm. This indicates that the concentration of the polymer was insufficient to enable the formation of consistent fibres and open network of scaffold.

The widest fibres in this series were produced by BMC2 with a diameter of 17.8 μm. This increase could be caused by the dramatic increase of viscosity and chain entanglement in this concentration range. Wider fibres however, reduced the pore size to 38.4 μm, meaning that more space between the fibres was taken up, which resulted in a denser mat.

The pore structure became progressively better at 20% and onwards. The increased viscosity may have produced more stable electrospinning jet and led to more uniform deposition of fibres. The optimum morphology was found in this series at the concentration of BMC5 (25% zein). This sample observed a fibre diameter of 15.6 μm and the largest pore size of 61.9 μm. In addition, it provided the most stable and reproducible processing behaviour. As a result, 25% zein was chosen as the operating reference concentration, as it provided an optimal combination of the fibre formation, process stability and pore architecture.



(a) (b)

(c)

**Figure 3.8** SEM micrographs of the BMR fibres taken for reproducibility check: a) BMR1, (b) BMR2, (c) BMR3

The repeatability phase aimed to assess whether the three different electrospinning runs (BMR1, BMR2 and BMR3) could be performed with the same fibre morphology and scaffold architecture at the optimized ethanol:water ratio of 70:30. The same fibre solution compositions and processing conditions were used to obtain the three replicates, which all yielded consistent ribbon-like fibres that did not bead, along with stable and continuous fibre topologies. These findings show that a stable and dependable electrospinning window is offered by the 70:30 solvent solution. Pore width of the three replicates varied from 56.1 to 70.8 μm and fibre diameter from 10.4 to 14.9 μm.

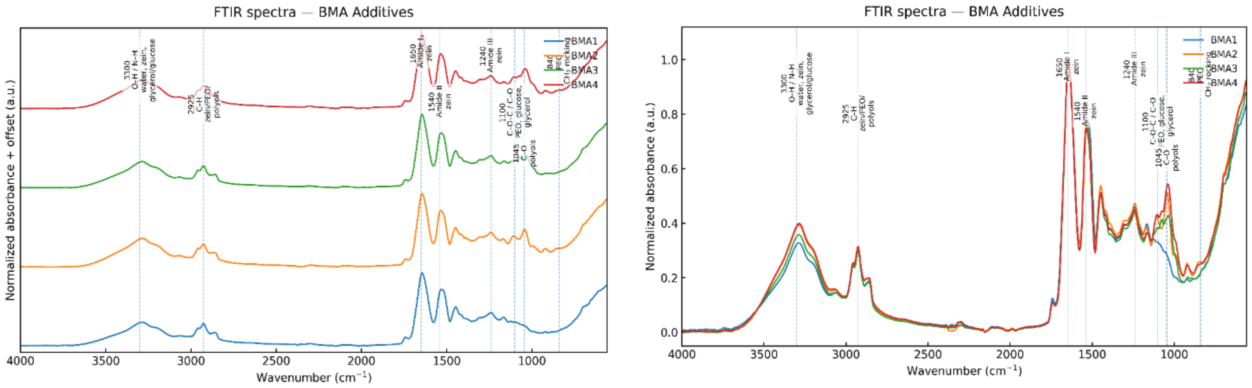
### 3.2. FTIR analysis and interpretation

Using a Bruker Vertex 70 spectrometer and Fourier transform infrared spectroscopy (FTIR), the chemical structure of the electrospun zein scaffold samples was examined. The spectrometer is capable of several modes of measurement such as attenuated total reflectance (ATR) which was selected for the present work as it is suitable for direct analysis of fibre mat samples containing solid protein without any additional sample treatment. It covers the spectral range from 400 $cm^{-1}$ up to 4000 $cm^{-1}$ and has a spectral resolution of up to 1 $cm^{-1}$. The fibre mats were dried at room temperature and then cut into proper size pieces for contact with the ATR crystal for good contact. Scaffold samples were also measured using the same measurement settings for each sample group: BMA (additive effect), BMF (flow rate influence), and BMS (solvent ratio screening) to allow for direct comparison of the measured spectra.

The amide I, amide II, amide III, and O–H/N–H bands of the main zein protein are seen in all three FTIR groups: BMA, BMS and BMF, at 1650 cm$^{-1}$, 1540 cm$^{-1}$, 1240 cm$^{-1}$, and 3300 cm$^{-1}$, respectively. These bands show that the zein is still the most abundant scaffold forming protein. The literature reports the amide I band at around 1650 cm$^{-1}$, the amide II band at around 1540 cm$^{-1}$ and the amide A/N–H band at around 3300 cm$^{-1}$ [75].

The aliphatic C–H stretching is found between 2850 and 2950 cm−1, which can be associated with zein side chains and may also be associated with additives such as glycerol, glucose and PEO. The polyol/ether-type additives, such as glucose, glycerol, and PEO, are supported by the presence of the strong fingerprint region in the spectrum between 1000–1150 cm$^{-1}$ which reflects the C–O and C–O–C stretching. The C–O stretching near ~1100 cm$^{-1}$ is often attributed to PEO, whereas the C–O stretching at ~1100–1038 cm$^{-1}$ in the glycerol-rich systems is attributed to glycerol [76].

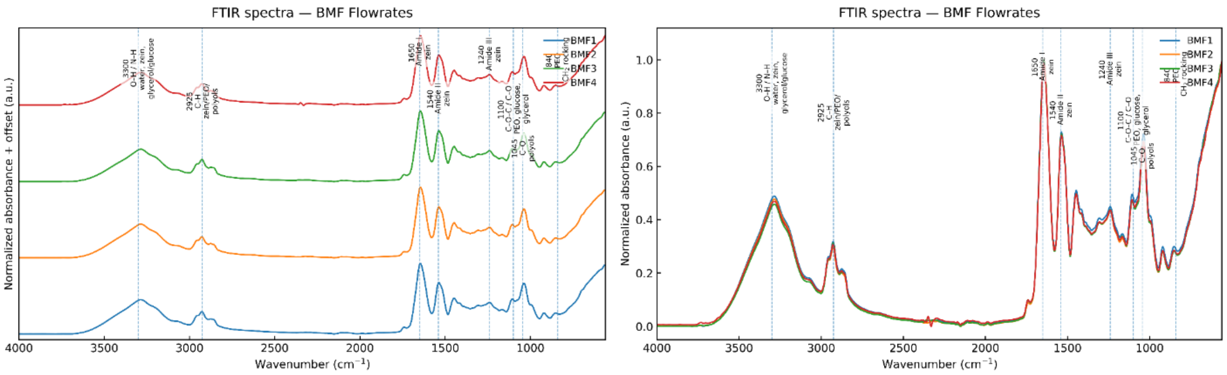
A wide range around 3200-3500 cm-1 can also be used for hydroxyl-containing species such as residual water/ethanol interactions, glycerol and glucose. This is however very close to zein N–H/O–H stretching and should, therefore, be looked at qualitatively, not quantitatively, as evidence for one entity [77]. FTIR is not a reliable method for identifying NaCl, as there are no characteristic mid-IR molecular vibrations of NaCl.



(a) (b)

**Figure 3.9** FTIR analysis of additive samples – BMA group

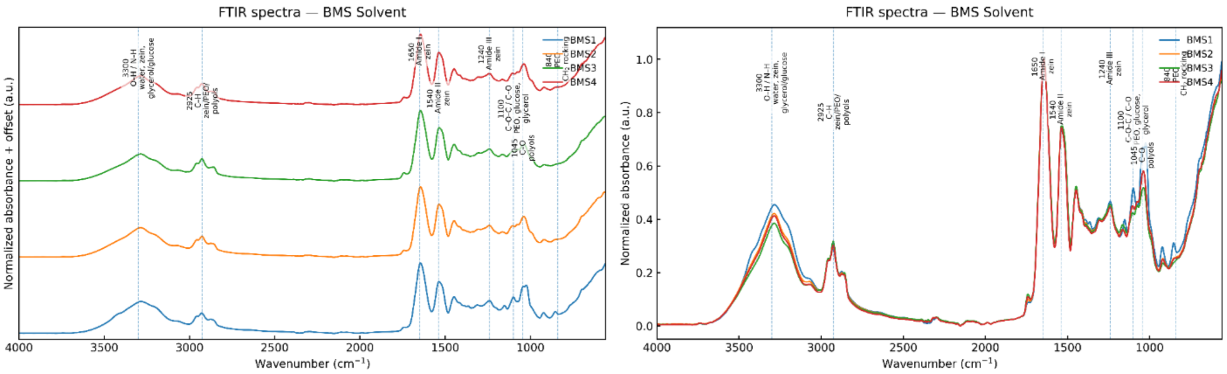
For the BMA additive group all samples contained the characteristic zein amide bands, indicating zein as the predominant scaffolding matrix. The spectra of BMA2, BMA3 and BMA4 revealed enhanced absorption around the O–H/N–H region of 3200–3500 cm$^{-1}$ and the C–O/C–O–C fingerprint region of 1000–1150 cm$^{-1}$ when compared to BMA1. These changes are in line with the addition of hydroxyl additives. BMA2 is largely associated with glycerol, BMA3 with glucose and BMA4 with a combination of both additives. FTIR is used for confirmation of the qualitative presence of glycerol and glucose but this is not done by completely separate unique peaks as the hydroxyl and C–O peaks are similar for both substances and can also be seen in other components of the sample.



(a) (b)

**Figure 3.10** FTIR analysis of flowrate samples – BMF (70/30) group

The FTIR spectra of the BMF flow-rate group samples were very similar. The zein amide bands are still present at ~1650, ~1540 and ~1240 cm-1 even with varying flow rates, as illustrated by the consistency in these bands for the different flow rates. The C–H stretching band around 2925 cm$^{-1}$ and the C–O/C–O–C region around 1000–1150 cm$^{-1}$ were also left, which means that the formulation with the additives was still basically the same in terms of chemistry. Thus, there is not a significant influence of flow rate on chemical composition, but its effect is likely to be primarily on fibre morphology, jet stability, fibre diameter and uniformity of the scaffold.



(a) (b)

**Figure 3.11** FTIR analysis of solvent ratio samples – BMS group

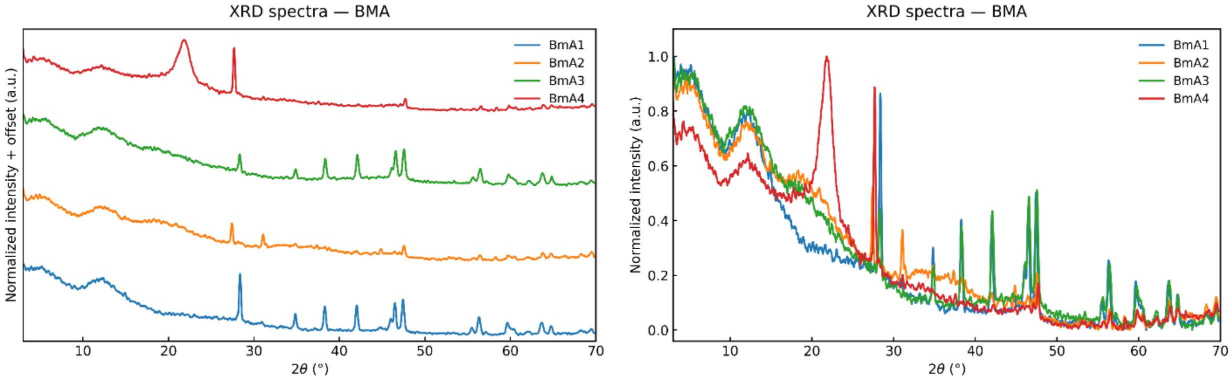
All the spectra in the BMS solvent-ratio group contained the characteristic zein amide I, amide II and amide III bands, showing that there was no significant chemical change of the zein protein matrix with variation in ethanol-water ratio. Main differences were detected in the broad 3200-3500 cm-1 region and the 1000-1150 cm-1 fingerprint region, which could be related to the changes of hydrogen bonding, residual water/ethanol interactions and distribution of hydroxyl-containing additives. Thus the ratio of solvent components was the most important factor affecting the physical fibre formation and molecular interactions, and not the presence or absence of the individual components.

### 3.3. XRD analysis and interpretation

The structural state of the electrospun fibre scaffolds made of zein was assessed by X-ray diffraction. The primary objective was to find out if the scaffolds were mainly amorphous or crystalline domains had formed as a result of additives, solvent composition or changes in flow rate.

Zein is a protein extracted from corn that has been approved for use in food products under proper purification conditions and it is generally applied in food films and edible coatings. Zein is listed in the FDA regulations at 21 CFR §184.1984 as being a component of corn gluten and that it must be of suitable purity for its intended use [78]. Thus, the scaffold is not inedible because of the presence of zein. But, in the case of scaffolds of cultured meat, a highly crystalline or residual additive-rich structure may not be desirable as it can impact digestibility, thermal behaviour, cell-scaffold interactions and hydration, and texture.

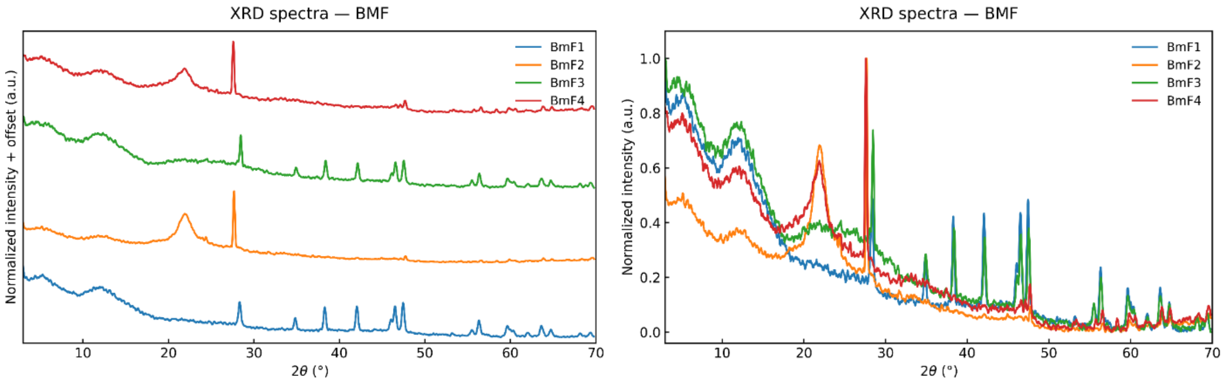
The XRD patterns show a very broad diffuse background for all of the groups, primarily in the lower 2θ region, from about 5–25°. This is a wide halo typical of an amorphous or poorly ordered polymer/protein matrix. In zein-based materials, broad XRD features are found to be correlated with the amorphous structure of zein, while sharp diffraction peaks are correlated with the crystalline structure [79].This indicates that the zein fibre network under the majority of the samples is not highly crystalline, which is desirable for an edible cultivated meat scaffold as amorphous protein-based matrices are typically softer, more hydrated and more texturally compatible with food. At the same time, several samples show sharp diffraction peaks, particularly around approximately 21.7–22.0°, 27.5–28.4°, 37–38°, 42–48°, 56–57°, and above 60°. The sharp peaks are associated with the presence of crystalline domains.



(a) (b)

**Figure 3.12** XRD analysis of additive samples – BMA group

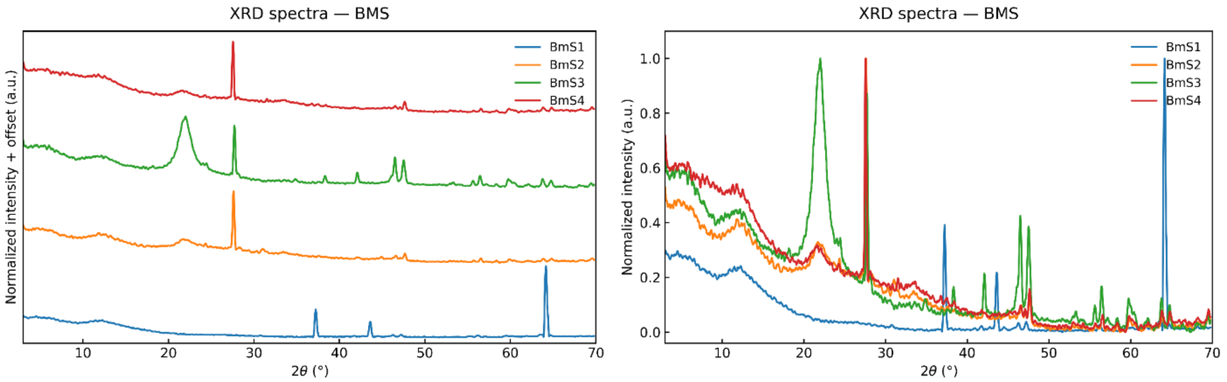
Additives have the most pronounced effect on crystallinity of the BMA group. The x-ray diffraction pattern of BMA1 and BMA3 have some sharp peaks in the high 2θ region; BMA4 has a strong peak at 21.8° and another intense peak at 27.7°. This suggests that partial crystallization or remaining crystals in the fibre mat might have been achieved by incorporation of the additives. This is relevant from a cultivated meat point of view. Ideally, a scaffold should be edible, hydrated, flexible and digestible. Hard ordered domains are indicated by sharp crystalline peaks, but this can have a negative impact on mouthfeel or thermal behaviour during cooking. The peaks that are observed may not necessarily reflect the presence of NaCl or PEO residues, but do indicate that washing, formulation or post-treatment optimization may be required.



(a) (b)

**Figure 3.13** XRD analysis of flowrate samples – BMF (70/30) group

Significant variation is seen between the solvent conditions for the BMS group. The broad zein halo is not as dominant in the BMS1 and sharp peaks can be observed at 37.2°, 43.6°, and above 60°. BMS2, BMS3, and BMS4 show stronger crystalline peaks around 21.7–22.0° and 27.6–27.7°. The structure is especially interesting in BMS3 as there is a strong peak around 22.0° indicating the presence of more ordered structure. This means that the ratio of ethanol water solvent has a significant influence on the organization of the fibre molecules. The rate of evaporation of the solvent, phase separation and the mobility of the polymer chains may affect the resulting zein structure (amorphous vs crystallization of additives during the drying process) in electrospinning process. Smoother fibres may result from a solvent system that will lead to crystallization which is not suitable for scaffolds used in edible cultivated meat.



(a) (b)

**Figure 3.14** XRD analysis of solvent ratio samples – BMS group

The BMF group also exhibits a variation in crystallinity as a function of flow rate. The sharp peaks in the region 38-48° are observed for BMF1 and BMF3, whereas BMF2 and BMF4 exhibit strong peaks around 21.8° and 27.6-27.7°. This indicates that there may be a dependence of flow rate on solvent evaporation and fibre solidification. A higher flow or a flow that is not stable can lead to increased residual solvent or migration of additives to the drying temperature during drying. Application of scaffolds would benefit from a flow-rate that yields a relatively broad amorphous peak with little sharp crystalline peak, which would indicate a more uniform zein-rich matrix.

Crystalline sharp peaks suggest the presence of ordered domains or additives that are still crystalline in the zein scaffold. Zein is food-compatible, but too much crystallinity could be undesirable for scaffolds for cultivated meat, as it might decrease flexibility, change hydration, or influence texture upon freezing and cooking. As a result, the formulations with mostly amorphous zein rich patterns that have fewer sharp crystalline peaks are better suited for scaffold development for edible applications.

### 3.4. Discussion

1. The solvent ratio was determined as the most important starting parameter for zein electrospinning for the experimental phases. Continuous ribbon fibres with scaffold architecture that was optimal for the continuous growing of ethanol-water ratio was achieved at 70:30 ratio. The results of solvent screening phase indicated that BMS2 had the most appropriate condition of solvent fibre diameter (10.1 µm) and pore size (64.2 µm). Hence most of the optimization steps were performed on the 70:30 solvent system as a reference.

2. The fibre diameter and pore architecture were also affected by flow rate, voltage, distance, additives and zein concentration. For 70:30 flow-rate series, pores with the highest openness (11.0 µm fibre diameter and 68.4 µm pore size) were obtained with 4 mL/h flow-rate. The effect of voltage was also found to be non-linear with fine, stable fibres (9.5 µm; 48.1 µm pore size) at 14 kV. The distance study revealed that 10 cm had the most open structure (11.1 µm; 77.1 µm pore size). When glucose was used alone, it resulted in compact fibre packing (11.7 µm; 44.2 µm pore size), and glycerol was used alone, it had better pore openness and stable processing (10.7 µm; 56.2 µm pore size). In zein concentration phase, zein 25% showed the maximum pore size (61.9 µm pore size; 15.6 µm fibre diameter) and was determined as the most appropriate concentration to manufacture stable scaffolds with zein.

3. FTIR confirmed the chemical preservation of zein protein matrix in all experimental conditions, and XRD showed that the majority of the samples had a predominantly amorphous structure, which are desirable properties of an edible scaffold. In selected samples (BMA4, BMS3) there are sharp crystalline peaks suggesting that formulation optimisation may be required prior to cell culture studies. The conditions identified in this study to obtain the optimum parameter set (25% w/v zein, 70:30 ethanol:water, 0.3% PEO, 0.5% NaCl, 7% glycerol, 10% glucose, 14 kV, 4–6 mL/h, and 10 cm) allow for a reproducible platform for scaffold fabrication suitable for future cell seeding experiments, while maintaining conditions compatible with food applications.

## 4. Recommendations part

The study demonstrates that electrospinning is a better method to produce zein scaffolds for cultivated meat. A systematic investigation of the composition of the solvents, processing conditions and additives was carried out, and a set of process parameters was identified that always produced stable fibres with acceptably good morphology and intact chemical structure. The conditions identified in this study to obtain the optimum parameter set (25% w/v zein, 70:30 ethanol:water, 0.3% PEO, 0.5% NaCl, 7% glycerol, 10% glucose, 14 kV, 4–6 mL/h, and 10 cm), were found to form ribbon-like fibres along with an open porous structure. The XRD results confirmed that the structure of the scaffold was amorphous and edible. The FTIR results suggest that the chemical integrity of zein was unaltered even after the electrospinning process.

The optimized scaffolds resulted from this experiments consists of a porous structure which is suitable for cultivated meat applications. The next step is to characterize the attachment, growth, differentiation and alignment of muscle satellite cells on these scaffolds. In addition, mechanical testing, degradation profiling and long-term stability testing under cell culture conditions would help to further elucidate the performance of the scaffolds in a real tissue engineering context. The recommendation for the future may be translating the complete production workflow, including the steps of solvent preparation, dissolution of zein into solvent, electrospinning, sterilization, and quality control as shown in the proposed production workflow diagram (Figure 4.1) on an industrial scale. While this study was performed on a lab-scale, it would be beneficial to investigate high throughput electrospinning platforms. The incorporation of solvent recovery systems would also have a major impact on consumption of ethanol and the environmental impact of large-scale ethanol production. However, based on the optimum parameters used here it appears that zein fibrous scaffolds can be made in a repeatable and feasible process that could be the basis for future cell-seeding studies and offers tremendous potential for the development of structured grown animal meat.

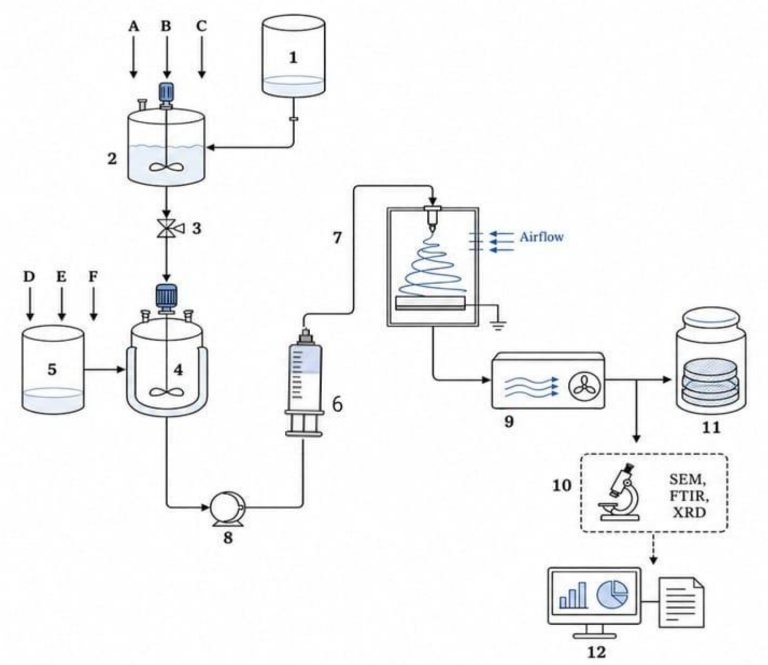


**Figure 4.1** Proposed technological scheme for the production of zein-based electrospun fibrous scaffolds for cultivated meat applications

**Table 4.1** List of equipments and materials

| **Symbol** | **Equipment / Material** |
|---|---|
| **1** | Solvent reservoir |
| **2** | Mixing tank with stirrer |
| **3** | Valve |
| **4** | Zein solution preparation reactor |
| **5** | Additive reservoir |
| **6** | Electrospinning feed reservoir |
| **7** | Electrospinning unit / collector chamber |
| **8** | Pump / syringe pump |
| **9** | Drying and conditioning unit |
| **10** | Quality control point |
| **11** | Final scaffold collection / storage |
| **12** | Data recording / reporting |
| **A** | Zein |
| **B** | Ethanol |
| **C** | Distilled water |
| **D** | PEO |
| **E** | NaCl |
| **F** | Glycerol / glucose |

## Conclusions

1. This literature review confirmed that conventional livestock production is not sustainable to provide protein for a future world population of over 9 billion by 2050, and the environmental, ethical and public health consequences of continuation on this path are not only undesirable, but essential for the need to find alternatives. Of all alternative protein solutions being developed, only cultivated meat has the potential to truly replicate the sensory and structural experience of the whole-cut meat. To do that, a scaffold is required and the review showed that the plant-based materials, especially zein, are the most promising materials to serve as a scaffold that is food-safe and sustainable.

2. With this as a scientific foundation, the experimental part focused on determining if the zein could be electrospun into a viable scaffold and under what conditions. Four combinations of ethanol:water were tested before it was realized that 70:30 was the right ratio. It provided the correct level of solubility, viscosity and surface tension to ensure smooth continuous and bead-free ribbon fibres of an open porous structure. The removal of the solvent was actually beneficial, with the introduction of glycerol both the process was more stable and the pores were more open; adding glucose alone tended to bring the fibres together. The solution at the highest concentration (25% zein) exhibited the most consistent behaviour and the most consistent results over repeated runs.

3. By tuning the process parameters, it was found that electrospinning is a system of connectedness. The flow rate of 4 mL/h resulted in the most open scaffold structure, 14 kV was an optimum electrostatic force that did not cause instability of the jets, and a distance of 10 cm between the needle and the collector produced the largest pores observed throughout the entire study. Most importantly, when the conditions were optimised and repeated three times without mix-ups, they were all found to be the same, which is of critical importance if this type of scaffold is ever to be produced reliably on a large scale.

4. Last but not least, the characterisation work concluded that the product being produced was indeed appropriate for the intended use. FTIR demonstrated that zein protein was not chemically changed during the electrospinning process under all electrospinning conditions, indicating that the thermal stress of the electrospinning process itself did not harm the zein protein. The X-ray diffraction results indicated that the scaffolds had a predominantly amorphous structure, which is quite desirable for soft, hydrated and digestible scaffolds. The conditions optimized in this study - 25% zein in an ethanol:water solvent of 70:30, glycerol and PEO and NaCl, and spun at 14 kV, 4-6 mL/h and 10 cm distance - provide a solid and repeatable baseline for further studies, and ultimately, whether muscle cells can grow, align and mature on these scaffolds as they would in a real cultivated meat product.

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