Development of Sustainable Konjac Glucomannan-Based Microcarriers for Cultivated Meat Production

By

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Bachelor of Technology, Punjab Engineering College, 2022

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Abstract

The global demand for sustainable alternatives to conventional meat production has promoted advancements in cultivated meat technologies, with scaffolding materials playing a key role in supporting cell growth and mimicking natural meat structures. This study investigates konjac glucomannan (KGM), a plant-based polysaccharide, as a biocompatible and cost-effective material for microcarrier generation in cell culture technologies. A method was developed to synthesize KGM hydrogels and fabricate microcarriers via controlled acidic degradation and crosslinking with epichlorohydrin (ECH) using a water-in-oil emulsion technique. The resulting microcarriers demonstrated excellent biocompatibility, mechanical stability, and a reticulated structure that may supports cell adhesion and proliferation, competing with conventional dextran-based microcarriers while offering cost and sustainability benefits. These findings highlight KGM's potential as a cruelty-free microcarrier material for cultivated meat production and other biomedical applications, supporting the objectives of ethical innovation and global sustainability.

Keywords: Konjac Glucomannan (KGM), Meat Cultivation, Microfluidics, Batch-Emulsion, Epichlorohydrin (ECH), Microcarriers, Plant-Based Materials

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This work would not have been possible without the collective efforts of these remarkable individuals, and I am forever indebted to them for their contributions to my academic and personal growth.

Chapter 1: Introduction

Growing populations and rising incomes in developing nations are driving an unprecedented increase in the demand for meat worldwide [1]. Traditional meat production does, however, provide several difficulties, such as greenhouse gas emissions, water and land use, environmental degradation, and moral dilemmas pertaining to animal care [2]. Cultivated meat, also known as lab-grown or cell-cultured meat, has emerged as a promising alternative that aims to address these issues [3][4]. This section explores a brief history on meat cultivation, its technological developments, and the challenges it faces as a potential solution to the environmental and ethical dilemmas of conventional meat production [5] [6].

1.1 Historical Background

The concept of cultivated meat was first introduced in the early 20th century [3], but it did not gain momentum until advancements in cellular biology and tissue engineering in the late 20th and early 21st centuries [3]. In 1931, Winston Churchill predicted the development of meat grown in laboratories, envisioning a future where only the edible parts of animals would be cultured without raising the entire animal [8].

The first significant breakthrough happened in 2013, when Dutch scientist Mark Post unveiled the world's first lab-grown burger [7]. Created from muscle cells derived from cows, the burger marked a drastic moment of change in cultivated meat research. Although its production cost was approximately $330,000 [7], it still demonstrated the feasibility of creating meat from animal cells without the need for slaughter. Since then, advancements in stem cell technology, bioreactor design, and scaffolding materials have significantly reduced costs and improved scalability, bringing the technology closer to commercial viability.

1.2 Science Behind Meat Cultivation

Meat cultivation involves producing meat without slaughtering animals [4], addressing ethical and environmental concerns, while meeting growing demands from the market. However, the science behind cultured meat is complex [4], requiring ingenious processes to ensure the final product closely resembles traditional meat while being free from chemicals and contaminants [4].

Cultivated meat is produced using a process that replicates the natural growth of muscle tissue in animals [9]. The process begins with the extraction of animal cells, typically stem cells or satellite cells, which have the capacity to proliferate and differentiate into muscle, fat, and connective tissues [10]. These cells are then cultured in a nutrient-rich growth media containing amino acids, vitamins, minerals, and growth factors to promote cell growth and proliferation [11].

To create a structured product that resembles conventional meat, cells are grown on scaffolds, which provide a three-dimensional framework that mimics the extracellular matrix in animals [12]. Bioreactors are used to scale up production, offering controlled environments for cell growth, such as temperature, oxygen levels, and nutrient supply. The result is a product that replicates the texture, flavour, and nutritional profile of traditional meat [13].

1.3 Challenges Faced by Scaffolding Technologies

Scaffold technology is one of the most critical yet challenging aspects of meat cultivation. Scaffolds provide the three-dimensional framework necessary for cells to grow, differentiate, and organize into structures resembling natural meat. However, selecting suitable materials for scaffold making is a major issue [14]. Scaffolds must be biocompatible, edible, and capable of supporting cell adhesion and growth while

replicating the texture and structure of conventional meat [14]. Balancing these properties with cost-effectiveness is difficult, especially at a larger scale. Additionally, scaffolds must enable adequate nutrient and oxygen diffusion to cells, which is typically achieved through vascularization in natural tissues. Without these features, cells in the interior of the scaffold may not survive, limiting the scalability of the process [15]. Moreover, achieving the mechanical properties required to replicate the texture of meats, such as steaks or chicken breasts, poses a greater challenge [9]. The integration of multiple cell types, such as muscle, fat, and connective tissue, adds further complexity, as scaffolds must support their simultaneous growth and interaction [16]. On top of these technical challenges, the environmental sustainability and regulatory approval of scaffolding materials remain a challenge. Many current materials, such as animal-derived collagen, contradicts with the ethical goals of cultivated meat, while synthetic options may face consumer skepticism [17].

These challenges emphasize the dire need for out-of-the-box approaches to advance scaffold technology. One promising approach is the use of microfluidics, which offers the potential to significantly enhance throughput and reduce costs.

1.4 History of Microcarriers

Microcarriers were introduced by van Wezel in 1967 which transformed cell culture by enabling anchorage-dependent cells to grow in suspension, overcoming the limitations of traditional monolayer systems [18]. These innovative tools provide a higher surface-to-volume ratio, scalability, and cost effectiveness, making them highly desirable for large-scale applications such as vaccine production, regenerative medicine, tissue engineering, and more recently, meat cultivation [19].

Initially, microcarriers were primarily composed of synthetic and semi-synthetic materials such as polyacrylamide, polyethylene glycol methacrylate (PEGMA), and gelMA [20], however, over time, advancements have led to the adoption of natural materials, including dextran, gelatin, cellulose, chitosan, and other complex polysaccharides [21][22][23]. While synthetic microcarriers offer precise control over mechanical and chemical properties, their production is often costly and puts toll on the environment [24]. In contrast, natural materials provide inherent biocompatibility and biodegradability, aligning with sustainable practices.

Despite these advancements, the high costs and environmental impact of traditional materials have driven the search for more sustainable alternatives. Among these, plant-based materials, particularly konjac glucomannan (KGM), have emerged as promising candidates [25]. Derived from the tubers of *Amorphophallus konjac*, KGM is a low-cost, abundant, and biodegradable polysaccharide with exceptional properties, including high viscosity, great gelation and excellent biocompatibility [26]. These characteristics make it an ideal substitute for conventional microcarriers in diverse applications.

KGM has demonstrated its versatility in biomedical fields such as stem cell proliferation, wound healing, and tissue engineering [27][28]. When combined with other materials like collagen or silk fibroin, it exhibits enhanced mechanical strength and bioactivity [29]. Additionally, its structural similarity to dextran and ease of chemical modification further expands its potential applications [30]. The transition to plant-based materials like KGM reflects a broader commitment to cost-effective, sustainable, and cruelty-free solutions, meeting both scientific and ethical goals.

1.5 Project Overview

In this project, I focused on developing a novel konjac glucomannan (KGM) hydrogel to produce microcarriers intended for cell culture applications. The hydrogel preparation presented some challenges due to the unique properties of KGM as a highly hydrophilic material [25][27]. Even at low concentrations (~0.5% w/w), KGM forms a highly viscous gel with limited flowability, making it difficult to handle and unsuitable for microcarrier generation [30].

Typically, hydrogels formed with other materials require a concentration of about 0.5-1% (w/w) [30], which ensures sufficient flowability and workability for downstream applications. However, KGM's viscous nature and limited solubility at similar concentrations create barriers to its direct use in microcarrier production [27]. Overcoming these challenges required careful optimization of the gel formulation and processing techniques which are discussed in the later sections.

Once the hydrogel was successfully prepared, a water-in-oil emulsion technique was employed to produce KGM microspheres [30][31]. The resulting microcarriers varied in diameter, ranging from 10 μm to 500 μm, making them suitable for diverse cell culture applications.

Chapter 2: Materials and Methodology

2.1 Materials

Konjac Glucomannan powder and Liquid Paraffin were purchased from Amazon.ca. Epichlorohydrin was purchased from Thermo Fischer Scientific Inc. Hydrochloride Acid, Sodium Hydroxide, Sodium Borohydride, Sodium Hydroxide Pellets,

Petroleum Ether were purchased from UVic Science Store. Other chemical reagents were available in Dr. Akbari's lab.

2.2 Review on Potential Materials for Microcarrier Generation

Throughout my research, I explored eighteen materials for microcarrier generation, including both animal-derived and plant-based options. Animal-derived materials such as gelatin, chitosan, collagen, and eggshell membrane are well-known for their excellent cell adhesion properties [32], which can support robust cell growth [33]. However, these materials come with ethical concerns surrounding animal cruelty. Additionally, certain proteins within the extracellular matrix of animal-derived materials have the potential to induce toxicity, which can lead to rejection by the cell lines they are intended to support [34]. Furthermore, once ingested, these animal-derived microcarriers can be rejected by the human body primarily due to immune system responses [34]. The degree of rejection depends on several factors, including the source and processing of the material, its structure, chemical properties and the host's immune system.

In contrast, plant-based materials are significantly less likely to be rejected by animal cell lines and/or the human body [25]. This is largely due to their structural and chemical composition, which aligns closely with naturally occurring compounds recognized by biological systems [27]. Plant-based materials are primarily composed of complex carbohydrates, such as cellulose and starch, as well as proteins, all of which are familiar to both animal cells and the human body [35].

The human body is equipped with the enzymes, antigens, and antibodies necessary to break down these plant-derived substances, making them inherently biocompatible. This natural compatibility reduces the likelihood of immune responses triggering

adverse reactions. Moreover, the human body's accustomed ability to metabolize and process these materials further enhances their acceptance, making plant-based materials a highly favourable option for applications in cultivated meat and other biomedical innovations [25].

Plant-based materials, including soy, zein, agarose, pea protein, and konjac glucomannan, present a compelling alternative to animal-derived counterparts [36]. While soy, zein, and pea protein are particularly well-suited for creating fibrous scaffolds [38], their use in microcarrier applications remains limited due to practical and executional challenges [37]. Konjac glucomannan, in contrast, is a polysaccharide that has been relatively less explored in the context of cell culture. Despite this, its hydrophilic nature, biocompatibility, and unique gelation properties position it as a promising candidate for microcarrier development [25]. These characteristics make konjac not only a sustainable choice but also a technically viable option for microcarrier generation.

Figure 1 shows a bar chart containing various materials along with the number of times they have been reported for creating microcarrier/scaffold.

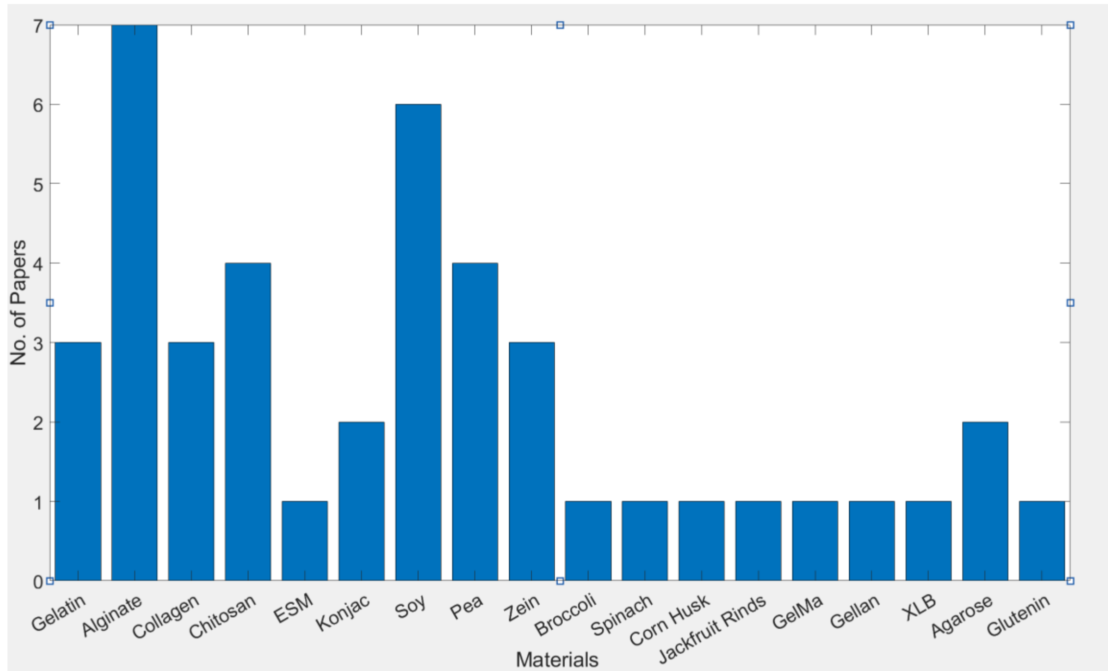

Figure 1: Plot between various animal/plant-based materials on X-axis and their respective number of articles available on Y-axis.

After reviewing 39 peer-reviewed papers on cell culturing materials and material coatings, I identified 21 material blends used in the fabrication of scaffolds and microcarriers. This library of data has been organized and presented in table 1, providing a detailed overview of the materials, material type, method of production and shape of microcarriers/scaffolds.

Table 1: Table lists all the possible animal/plant-based materials used for cell culturing applications along with suitable shape and method of producing scaffold/microcarrier respectively.

Material Type	***Material***	***Shape of Scaffold/Microcarrier***	***Method of Production***
Animal-Based	*Gelatine*	*Spherical*	*Water-in-Oil Emulsion*
Animal-Based	*Chitosan + Collagen + Oleogel fat blend*	*Spherical*	*Electro spraying*

Animal-Based	*Chitosan + Collagen Composite*	*Spherical*	*Electro Spraying*
Animal-Based	*Turkey Collogen + Eggshell Membrane*	*Spherical*	*Freeze Drying*
Animal/Plant - Based	*Gelatine, Soy, Glutenin, Zein, Cellulose, Alginate, Konjac, Chitosan*	*PDMS Mold*	*Molding*
Plant-Based	*Soy Protein*	*Porous Scaffold*	*Extrusion + Rehydration*
Plant-Based	*Zein*	*Film with Pores, Spherical*	*Purchased*
Plant-Based	*Konjac Glucomannan (KGM)*	*N/A*	*N/A*
Animal/Plant-Based	*Salmon Gelatin, Alginate, Agar, Agarose, Glycerol*	*Porous Scaffold*	*Freeze Drying*
Plant-Based	*Agarose, Gellan, Pea, Soy, Xanthan Gum Locust (XLB)*	*Leachates*	*Mixing*
Plant-Based	*Pea Protein Isolates (PPI), Soy Protein Isolates (SPI)*	*Stranded Scaffold*	*FRESH Printing*
Plant-Based	*Alginate*	*Fibrous Scaffold*	*Wet Spinning*
Semi-Synthetic	*GelMA*	*Scaffold with Microchannels*	*DLP Printing*
Plant-Based	*Zein Coated Alginate Fibers*	*Stretchable Bands*	*Wet Spinning*
Plant-Based	*Corn Husk, Jackfruit Rinds*	*Geometric Shapes like Circle, Square and Triangle*	*Shaping*

Plant-Based	*Pea Protein Isolate (PPI), Alginate*	*Mold*	*Casting, FRESH Printing*
Plant-Based	*Soy Protein + Amyloid Fibrils*	*Mold*	*Casting*
Plant-Based	*Broccoli Florets*	*Florets*	*Sieving*
Plant-Based	*Decellularized Spinach Leaves*	*Circular Punches*	*Freeze Drying*
Plant-Based	*Soy Conglycinin, Chitosan*	*N/A*	*N/A*
Fungi	*Inactive Mycelium Biomass*	*Spherical Pellets*	*Pellet Formation*

2.2.1 Review on Konjac Glucomannan (KGM)

Konjac glucomannan (KGM) is a natural polysaccharide derived from the tubers of *Amorphophallus konjac*, characterized by a backbone of β-1,4-linked D-mannose and D-glucose in a molar ratio of 1.6:1 [25][27]. The structure also features a low degree of acetylation, contributing to its remarkable hydrophilicity and gel-forming properties. This molecular composition shares similarities with dextran [30], another commonly used polysaccharide in microcarrier applications, but KGM offers distinct advantages in cost and functionality [30].

2.2.2 Advantages of KGM Structure

The distinct characteristics of KGM is its high molecular weight and viscosity, which enables it to create strong and stable hydrogels at low concentrations [25]. In contrast to other plant-based materials like soy and pea isolates, KGM can achieve gelation without requiring substantial modification, making it easier to process [39]. Additionally, its biocompatibility and biodegradability present it as a sustainable option

for cell culture applications. Chemical alterations, such as amination with diethylaminoethyl (DEAE) groups, improve its cell adhesion abilities by adding a positive charge (-NH_4 group) that enhances interactions with negatively charged cells [27].

2.2.3 Comparison between KGM and Commercial Microcarriers

KGM stands out when compared to commercial microcarriers like Cytodex and dextran-based alternatives [30]. Cytodex-1, a widely used microcarrier, utilizes dextran as its base material and is modified with DEAE groups for cell adhesion [27]. While effective, Cytodex-1 is expensive due to the production and sourcing costs associated with bacterial-derived dextran. In contrast, KGM is derived from a high-yield plant source, making it more affordable [40]. For example, the cost of KGM powder is approximately one-tenth that of dextran powder, with even greater savings compared to processed dextran hydrogels [30].

Additionally, KGM microcarriers offer superior performance in some applications. Studies have shown that cells cultured on KGM microcarriers exhibit faster adhesion and higher growth rates compared to Cytodex-1 [30]. In specific cases, such as mesenchymal stem cell (MSC) proliferation, KGM microcarriers with optimized stiffness achieved 1.7 times better proliferation rate and significantly enhanced differentiation potential compared to Cytodex-1 [27][30].

2.3 Material Preparation

Given the uniquely long branchy molecular chains, which allows KGM to form thick viscous gel, it becomes important to pre-treat the KGM powder before it is employed for gel making [30]. The polysaccharide chains of KGM needs to be broken down to smaller chains, without altering its chemical structure. Therefore, degradation of KGM

is a critical step in modifying its physical properties for gel making, such as viscosity and molecular weight, to facilitate its application in microcarrier generation [25][27][30].

Controlled degradation is typically achieved through acidic hydrolysis to overcome these challenges. Degradation is also done to completely dissolve the powdered konjac without leaving any undissolved residues [30]. Figure 2 shows the flowchart of microcarrier generation from the KGM powder.

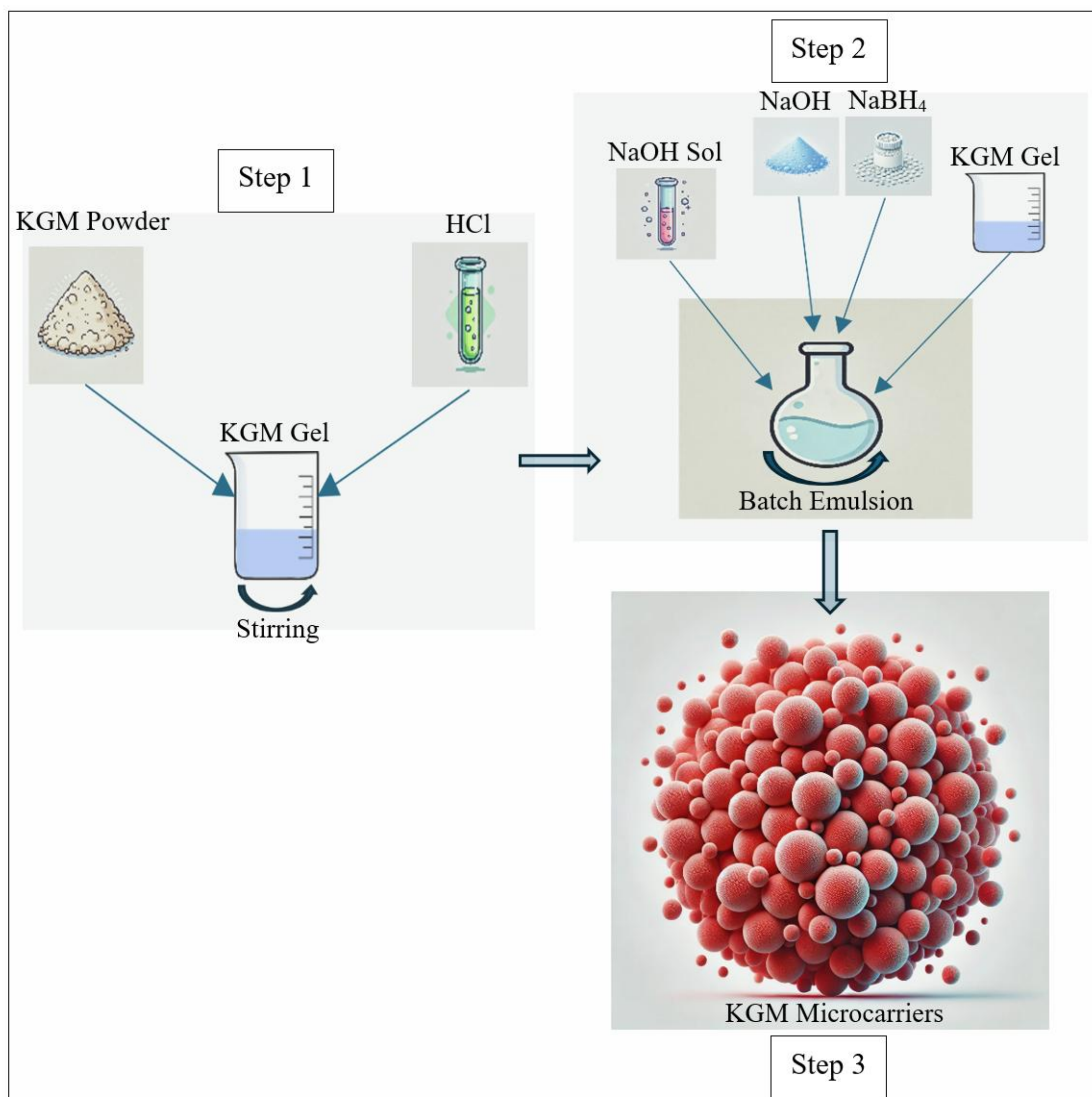

Figure 2: A detailed flowchart of KGM microcarrier production from KGM powder and other respective reagents.

2.3.1 Degradation of KGM

To degrade konjac glucomannan (KGM) while preserving its structural integrity, 1 g of pure KGM powder (containing ~90% glucomannan content) was dissolved in 50 mL of 0.5 M hydrochloric acid (HCl). The mixture was heated at 115°C for 55 minutes to break down the long polysaccharide chains, thereby reducing the molecular weight of KGM without compromising its essential properties [30].

Setup:

The setup consisted of a beaker containing KGM and HCl solution, placed in a water bath maintained at 115°C. A thermometer was used to continuously monitor the water bath temperature, as the degradation process is highly sensitive to temperature variations. The setup was eventually covered with silver foil to prevent the loss of water in both water bath and KGM + HCl solution.

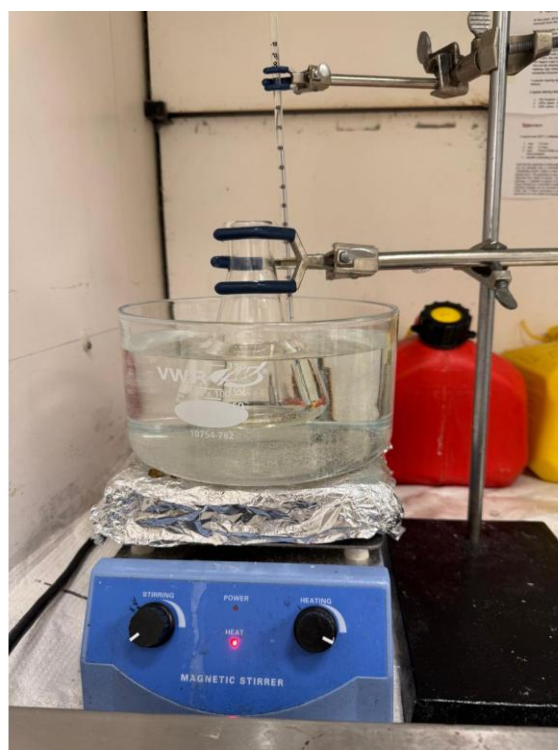

Figure 3: Figure shows the experimental setup created for the acidic degradation of KGM powder.

2.3.2 Preparation of KGM gel

Following the successful acidic degradation of KGM, a separate basic solution was prepared by dissolving 1 mg of sodium borohydride and 4 mg of pure sodium hydroxide (NaOH) crystals in 50 mL of 0.5 M NaOH at room temperature. This basic solution was then gradually added to the degraded KGM solution under vigorous stirring. The mixture was stirred continuously for 30 minutes to ensure complete neutralization and stabilization of the degraded KGM solution.

Discussion:

The addition of sodium borohydride serves to neutralize impurities in the KGM solution, such as ketone and aldehyde groups, which could adversely impact the gel's crosslinking efficiency [41]. These groups react with the epichlorohydrin (ECH) (crosslinker), reducing its availability for effective crosslinking. By targeting and neutralizing these impurities, sodium borohydride ensures that the majority of the crosslinker is utilized in forming the cross linkages within the gel matrix, thereby enhancing the structural integrity of the resulting KGM hydrogel [41].

The addition of excess NaOH serves to create an alkaline environment essential for the crosslinking reaction [42]. Adding 4 mg of NaOH to 100 mL of a neutral solution raises the pH from 7 to 11 approximately. This alkaline condition ensures that the epoxide ring in epichlorohydrin is activated, facilitating its opening and making it reactive. Once activated, the epoxide readily forms covalent bonds with hydroxyl (-OH) and carboxylic (-COOH) groups present in the KGM structure [43]. This reaction is critical for establishing a thorough crosslinked network within the KGM hydrogel.

Setup:

The neutralized KGM solution is contained in a beaker and vigorously stirred (2000-3000 rpm) at room temperature for 30 minutes, to form 1% (w/v) KGM gel.

Figure 4: Figure shows experimental setup carried out to neutralize the degraded KGM solution.

2.3.3 Formed KGM gels

By varying the concentrations of KGM powder, HCl, and NaOH, multiple gel samples were successfully produced using the same process. These variations resulted in gels tailored for different applications. Gels, which were highly dense (rubber-like consistency), exhibited properties suitable for injection molding, while thinner gels (water-like consistence) were optimized for microfluidic or batch emulsion applications [30]. Across all cases, the KGM gels showed exceptional stability under diverse conditions, including room temperature, elevated temperatures, and colder environments, unlike the competitors.

Figure 5 shows the gel consistency with varied concentrations of KGM, HCl and NaOH.

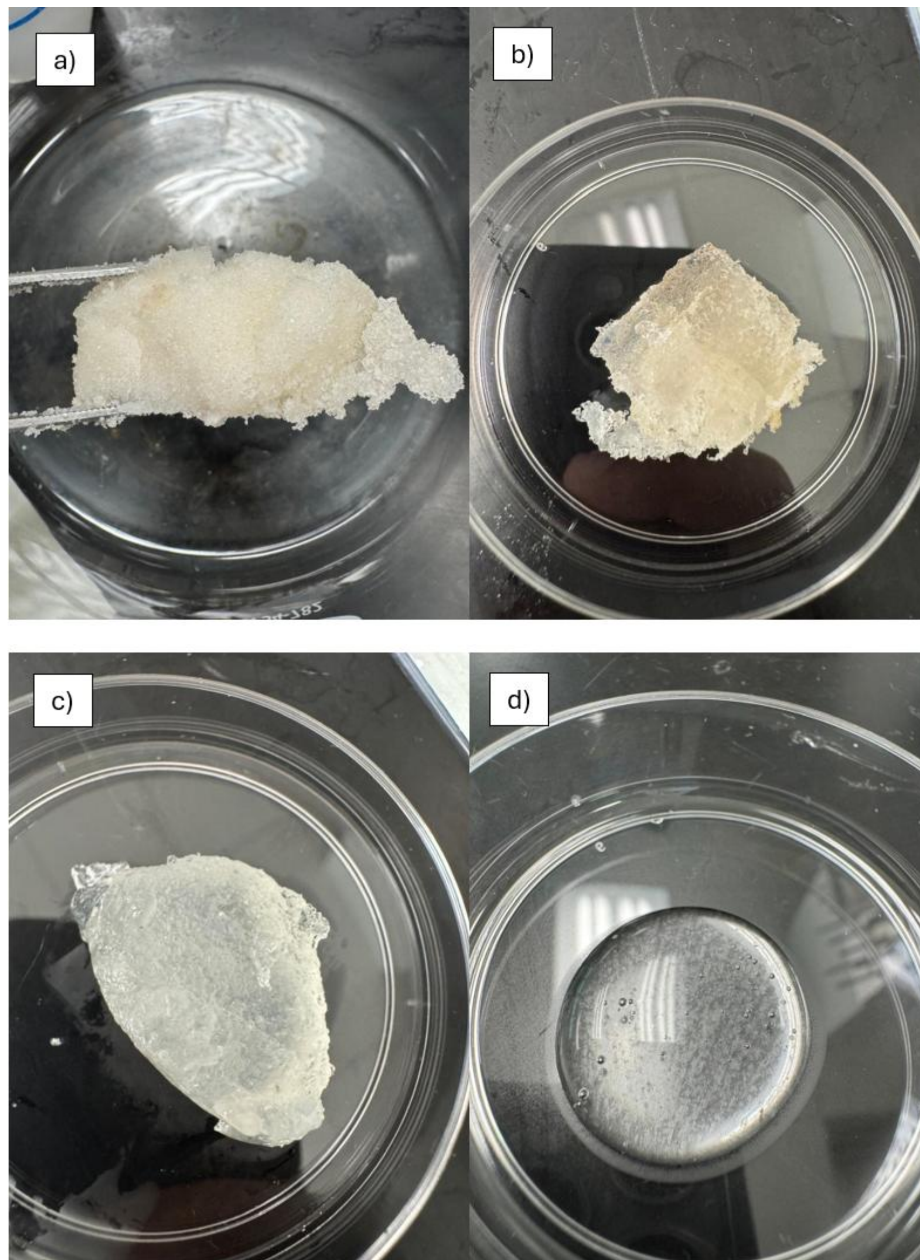

Figure 5: Figure shows gel consistence with varying the concentration and amount of KGM powder, HCl, NaOH. Pannel (a) shows 50% (w/v) KGM gel. Pannel (b) shows 20% (w/v) KGM gel. Pannel (c) shows 10% (w/v) KGM gel. Pannel (d) shows 1% (w/v) KGM gel.

2.4 Preparing Microcarriers

Producing microcarriers is a complex process that demands a deep understanding of the materials involved. There are several techniques available for making microcarriers,

and the choice of method depends on factors such as desired size and production throughput [44].

For high-throughput production, microfluidics is an excellent option [45]. This technique allows precise control over the size of microcarriers by varying the design of the microfluidic chip. Additionally, microfluidics is particularly well-suited for creating encapsulated microcarriers [46]. However, drawback of microfluidics is that it requires a large number of equipment like hydraulic controller, syringe, microfluidic chip, microchannels, UV light setup etc, and is a time consuming process.

In this project, batch emulsion was chosen to create microcarriers because of its ability to produce uniform, spherical particles with a controllable size range [30]. This method involves dispersing a water phase containing KGM gel into an oil phase while constantly stirring the mixture. The process forms stable microspheres, which are simultaneously crosslinked to achieve the desired mechanical strength and structural integrity.

2.4.1 Oil Phase for Batch Emulsion:

The choice of oil plays a significant role in deterring the efficiency of the process, as it must effectively shear the gel droplets from the aqueous phase to form stable microspheres. Additionally, the oil must be compatible with the chosen crosslinker to ensure proper crosslinking and stabilization of the microcarriers.

There is a wide variety of oils available to be used in batch emulsion, ranging from heavy mineral oils to plant-based and synthetic oils. The selection depends on factors such as viscosity, polarity, and interaction with the aqueous phase. For this project, the relatively high viscosity of the KGM gel, measured at approximately 362 mPa·s at 35°C, calls for a higher viscosity oil phase to ensure efficient emulsification [30]. The

viscosity of the oil should complement the gel's properties, i.e., the viscosity of the oil phase should be higher than that of the aqueous phase, allowing for the formation of uniform droplets.

In this project, two oils were utilized to create the oil phase: liquid paraffin (LP) and petroleum ether (PE) [48]. These oils were chosen for their complementary properties, which facilitated the emulsification process and contributed to the effective formation of microcarriers. To further optimize the system, span 80 (S8) was employed as an emulsifier (surfactant) [30].

The use of an emulsifier in batch emulsion is essential for ensuring the stability and quality of the resulting microcarriers. Span 80, a non-ionic surfactant, plays a critical role in preventing the microcarriers from aggregating or attaching to each other during the emulsification process [47]. This is achieved by reducing the surface tension between the aqueous and oil phases, stabilizing the dispersed droplets, and forming a protective layer around the microspheres. The addition of the emulsifier ensures that the microcarriers remain distinct and maintain their uniformity in size and shape throughout the process.

Setup:

For optimal results in this project, the ratio of the oil phase to the aqueous phase was determined to be 100:10 (v/w). This means that for every 100 mL of the oil phase, 10 grams of the aqueous phase containing KGM gel was used. This ratio was chosen to achieve a balance between efficient emulsification and the stability of the resulting microcarriers.

The composition of the oil phase was also precisely optimized to enhance its effectiveness in the batch emulsion process. Liquid paraffin (LP), petroleum ether (PE),

and Span 80 (S8) were combined in specific proportions: 80% of the total oil phase volume was liquid paraffin, 15% was petroleum ether, and the remaining 5% was Span 80. These percentages were carefully selected after carrying out a multitude of experiments.

To prepare the oil phase, 80 mL of liquid paraffin and 15 mL of petroleum ether were measured and combined in a clean beaker. Following this, 5 mL of Span 80 was added to the mixture. The components were thoroughly stirred for 30 minutes to ensure the formation of a homogeneous oil phase. Throughout the process, the mixture was maintained at a temperature of 60°C to enhance the uniformity and stability of the oil phase [30].

2.4.2 Aqueous Phase for Batch Emulsion

In the batch emulsion process, the aqueous phase consists of a hydrogel mixed with a crosslinker in calculated proportions. The ratio of hydrogel to crosslinker is critical to ensure complete crosslinking of the hydrogel, leaving no excess crosslinker in the system. This precision is essential because the mechanical stiffness and structural integrity of the microcarriers directly depend on the amount of crosslinker used [27][30]. Choosing the correct ratio not only enhances the mechanical properties of the microcarriers but also ensures biocompatibility. Any residual crosslinker left at the end of the process can introduce toxicity, which may hinder cell growth or compromise the viability of the microcarriers [49].

In this project, epichlorohydrin was the choice of crosslinker as it is the most widely used crosslinking reagent for polysaccharides [42]. Konjac being a natural polysaccharide, is a notoriously viscous material and hence requires a stronger

crosslinking reagent like epichlorohydrin over others like borate, formaldehyde, glutaraldehyde and acetic acid [43].

Setup:

The aqueous phase for this project was prepared using 6.66 g of 1% KGM gel and 50% of 99.87% ECH (w/w of KGM gel), which equated to 3.33 g.

To prepare the mixture, 6.66 g of KGM gel was transferred into a falcon tube, followed by the addition of 3.33 g of ECH. The contents were then thoroughly shaken to ensure uniform mixing and create a homogeneous aqueous phase.

2.4.3 Batch Emulsion

The aqueous solution, comprising the KGM gel and ECH, was transferred into a 25 mL beaker and then gradually added dropwise into the prepared oil phase (containing LP, PE, and Span 80) over a period of approximately 30 minutes.

As the two phases mixed, the colour of the emulsion changed from clear to light pink. This colour change indicated that the crosslinker was being activated by the alkaline conditions of the aqueous phase [50]. The gradual addition of the aqueous solution allowed for controlled emulsification and ensured uniform droplet formation.

Setup:

The emulsion was allowed to react continuously for 8 hours at a temperature of 60°C, with constant stirring at a rate of 200-300 rpm [30]. Maintaining a consistent stirring speed was important, as the size of the microcarriers in batch emulsion is determined by the shearing action of the stirrer, unlike microfluidics where droplet size is defined by the chip design.

For this project, the stirring rate of 200-300 rpm was selected to produce relatively larger droplets, resulting in microcarriers with sizes ranging from 10 to 500 µm, suitable for cell culturing applications as it requires a larger surface area to seed cells. However, for applications demanding smaller droplet sizes or even nanocarriers, the stirring speed can be increased. By raising the rpm to a range of 1200-2000, it is possible to generate nanocarriers due to the higher shearing force breaking the droplets into much smaller sizes.

Chapter 3: Results

3.1 Gel Crosslinking

Before adding ECH into the batch emulsion, it was important to evaluate the efficacy of the crosslinking process. Without prior confirmation on the extent of its effectiveness on KGM, using ECH could introduce unnecessary toxicity. To establish the crosslinking efficacy, preliminary tests were conducted in two separate 25 ml beakers.

In one beaker, excess ECH was used (10 ml ECH + 5 ml KGM gel), while in the other, excess KGM gel was used (5 ml ECH + 10 ml KGM gel). The extent of crosslinking was consistent in both cases. However, in the beaker with excess ECH, some unreacted ECH remained, whereas in the beaker with excess KGM gel, all the ECH was utilized in the crosslinking process.

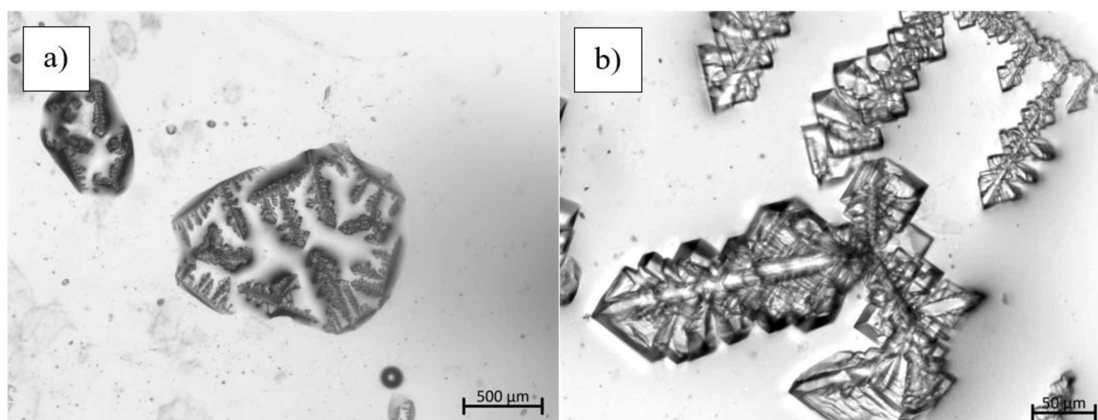

Figure 6: Figure shows a portion of the KGM gel getting crosslinked by ECH with the formation of crystals. (a) Gel crosslinking at 500 µm scale bar. (b) Gel crosslinking at 50 µm scale bar.

Figure 6 illustrates the crystal formation in KGM gel upon the addition of ECH. The reaction kinetics were slow and pH sensitive. However, after approximately 3 hours, the crosslinking process initiated crystal formation, which significantly altered the gel's viscosity. The transition from a viscous gel to a rubber-like scaffold was observed.

Crosslinking also modified the mechanical properties of the gel, including its flowability and elasticity. The gel's flowability decreased to zero, transforming it into an opaque leachate, as depicted in figure 7.

Figure 7: Figure shows KGM gel leachate formed by the action of ECH in a petri dish.

3.2 Formed Microcarriers

After an 8 hours long continuous batch emulsion reaction (mentioned in section 2.4.3), reaction flask was removed from the stirrer and inspected for formed emulsion. As expected, a cloudy clump (sediments) of the aqueous phase was formed at the bottom of the flask, separating it from the oil phase. This segregation of the aqueous phase from the oil phase was indictive of the microcarrier formation. A clear phase separation between the aqueous and the oil phase can be seen in figure 8.

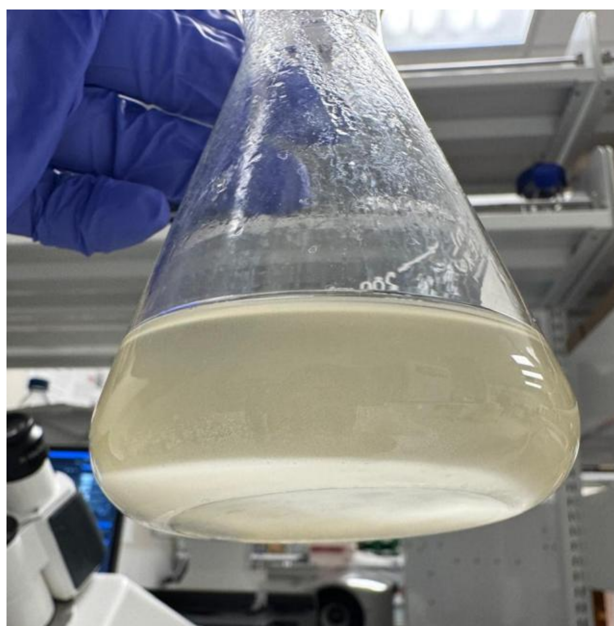

Figure 8: Formation of water-in-oil emulsion with KGM gel in aqueous phase and LP+PE in oil phase. Phase separation shows formed microcarrier's clump at the bottom of the flask.

A small portion of the sediment was carefully pipetted onto a petri dish and examined under a confocal microscope. The resulting images, as shown in figure 9, immediately revealed the presence of thousands of microcarriers within just a few drops of the sediments. It is very clear from the figure, that the emulsion contains a wide range of sizes of the microcarriers, which is one of the highlighting characteristics of batch

emulsion. The observable mean diameter of the KGM microcarriers is approximately around 50 µm, with sizes ranging from 10 µm to 500 µm.

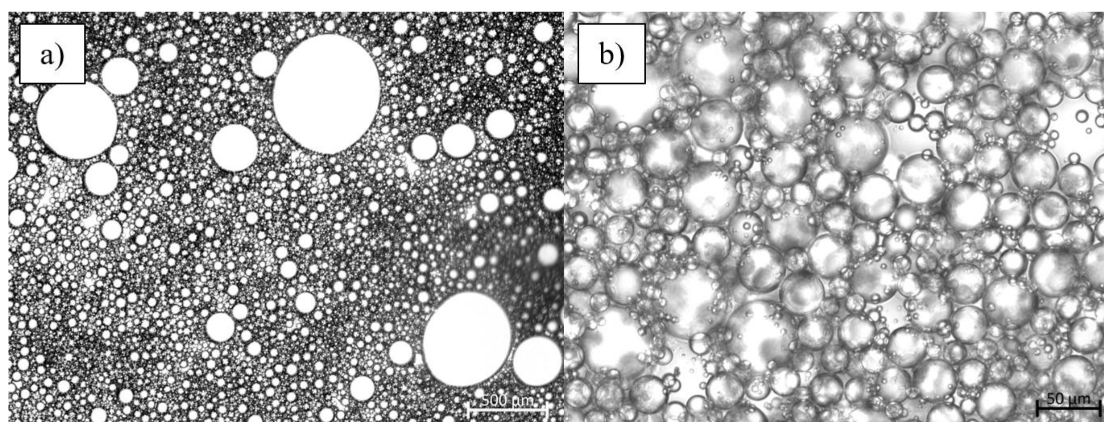

Figure 9: Confocal imaging of KGM microcarriers in a petri dish. (a) Confocal imaging at 25X magnification. (b) Confocal imaging at 200X magnification.

The KGM microcarriers closely resemble the commercially available dextran-based microcarrier, sephadex G25. However, notable differences exist in their surface morphology [30]. KGM microcarriers exhibit a reticulated structure with small interstitial spaces formed between KGM chains during the crosslinking process. In contrast, sephadex G25 features a drape-like surface morphology with visible fractures on its surface [30].

The unique reticulated structure of KGM microcarriers enhances their suitability for the fractionation of globular proteins within the molecular weight range of 1-10 kDa [30], making them a promising alternative to sephadex G25. Figure 10 provides a close-up view of the KGM microcarrier, highlighting details of its surface morphology. However, due to the limitations of confocal imaging, the reticulated structure is not clearly visible.

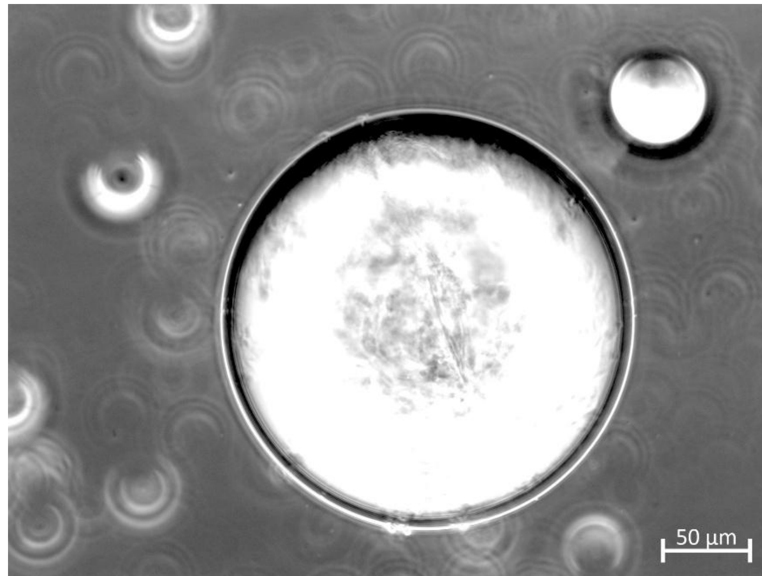

Figure 10: Image shows KGM microcarrier's surface morphology. The diameter of the microcarrier is roughly around 200 µm.

3.3 Failed Experiments

The outcomes of this project were highly promising, showcasing the potential of KGM with respect to alternatives to commercially available materials for cell culturing. However, achieving these results was not without challenges.

Throughout the process, there were several unsuccessful attempts, including failed gels, microcarriers that did not form as intended, and protocols that led to inconclusive or inaccurate results [30]. While these setbacks were a natural part of the experimentation process, each provided valuable insights that informed subsequent efforts.

This section documents some of these failed experiments, highlighting the iterative nature of scientific research and the role of challenges in driving progress.

3.3.1 Failed KGM gel

The absence of a well-defined protocol for preparing KGM gel often resulted in multiple unsuccessful attempts. The most referenced protocol [30], consistently

produced unsatisfactory outcomes, frequently yielding unusable gels. Figure 11.a illustrates an example of this issue, showing a KGM gel prepared using a (4:3 w/w) ratio of KGM to HCl.

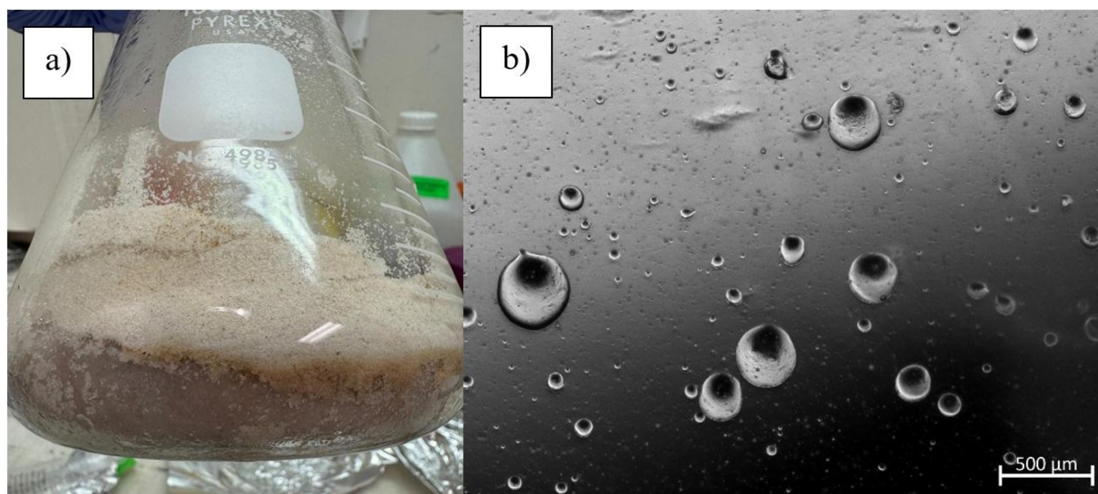

Figure 11: (a) Shows KGM gel stuck at the bottom of a flask. (b) Shows water droplets in oil phase deceiving as KGM microcarriers.

3.3.2 Failed KGM Microcarriers

Figure 11.b presents a misleading image of a batch emulsion, depicting spheroid-like structures that resemble microcarriers. These structures are, in fact, water bubbles trapped in the oil phase. Unlike microcarriers, which should remain intact, these water bubbles tend to merge with one another due to surface tension effects. This highlights the importance of carefully distinguishing between actual microcarriers and artifacts during analysis.

3.3.3 Gel Composition That Didn't Work

Table 2: Table contains various gels with varying the composition of the constituents.

Experiment No.	*Constituents*	*Comments*
1	*10g KGM + 20ml 0.1M NaOH + 40ml 0.5M HCl*	*Gel Failed*

2	*1g KGM + 20ml Water*	*Gel Failed*
3	*1g KGM + 40ml Water*	*Gel Failed*
4	*1g KGM + 60ml Water*	*Gel Failed*
5	*1g KGM + 80ml Water*	*Gel Failed*
6	*1g KGM + 100ml Water*	*Gel Failed*
7	*1g KGM + 140ml Water*	*Gel Failed*
8	*1g KGM + 160ml Water*	*Correct gel consistency but partially dissolved*
9	*1g KGM + 135ml Water + 25ml 12.18M HCl + 25ml 12.18M NaOH*	*Correct gel consistency*
10	*1g KGM + 185ml Water*	*Correct gel consistency but partially dissolved*
11	*1g KGM + 50ml 0.5M HCl + 50ml 0.5M NaOH*	*Correct gel consistency (Chosen Gel)*

4. Discussion

4.1 Discussion

This project demonstrates the significant potential of konjac glucomannan (KGM) as a sustainable, biocompatible, and cost-effective material for microcarrier production, addressing critical challenges in cultivated meat and other biomedical applications. The material's feasibility as a substitute for traditional dextran-based carriers is demonstrated by the successful synthesis of KGM hydrogels and microcarriers using batch emulsion [30]. KGM is reasonably priced because it is only a tenth of the cost of

commercially available Cytodex microcarriers. The distinct reticulated structure, excellent mechanical properties, and biocompatibility of the KGM microcarriers underscore their utility in supporting cell adhesion and proliferation [30]. Furthermore, this work lays the foundation for future advancements, such as surface amination to enhance cell adhesion and the integration of microfluidic systems to streamline production. These innovations create way for scalable, ethical, and environmentally friendly solutions in cultivated meat and beyond.

4.2 Future Work

4.2.1 Amination of Microcarriers

Despite its numerous advantageous properties, KGM has one limitation: its electrically neutral nature, which may reduce its efficiency in supporting cell adhesion. To enhance the surface adhesion of cells, KGM microcarriers can be modified through a process called amination, which introduces positively charged amine ($-NH_4^+$) groups. The positively charged KGM microcarriers can then interact with the typically negatively charged extracellular matrix (ECM) of cell lines, forming electrostatic bonds and significantly improving cell adhesion. Among the available reagents for amination, I plan to use 2-diethylaminoethyl hydrochloride due to its compatibility and effectiveness with KGM [27].

4.2.2 Creating Microfluidic Chip

The successful formation of KGM microcarriers opens the way for significant advancements, including the potential integration of microfluidic chips for their production. Utilizing microfluidic chips would not only enhance the throughput of the process but also make it more systematic, enabling precise control over the size of the microcarriers. A logical next step would involve designing an optimized microfluidic

chip capable of handling flows of oil, aqueous solutions, and ECH to facilitate the efficient and scalable production of KGM microcarriers.

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